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# PROGRESS IN BIOPHYSICS

## AND BIOPHYSICAL CHEMISTRY

## 5

*Editors*

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# EXTRACT FROM PREFACE TO VOLUME 1

THE editors have had some difficulty in deciding what is the proper field to be covered by reviews of recent progress in biophysics. Excluding biochemistry on the one hand and physiology on the other, there lies between a vast and rather amorphous field of study of which the frontiers and lines of demarcation are anything but well defined. On the one hand there is the application of the methods of physics to the study of the characteristic molecules of life—proteins, nucleoproteins, lipoproteins and other complex molecules of many and mostly unexplored varieties, a study which should perhaps be strictly regarded as an aspect of biophysical chemistry—hence the sub-title of the book. On the other hand there is the application of physical methods and instruments to the study and elucidation of living structures such as nerve and muscle fibres, a field in which modern refinements offer the greatest promise of progress in the future; progress which will perhaps bring its subjects into the first category. Both these fields of work are represented in the present volume and the editors have endeavoured to maintain a reasonable balance between them.

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It has been our aim to obtain readable and interesting reviews of recent progress in selected subjects. Our contributors have been asked not merely to compile a précis of recent papers, or annotated lists of references, but to write critical reviews in which they discuss the subjects from their own point of view; which may be read with profit by many who are not experts and which will provide scientists with a general survey of recent work and ideas.

Since it is impossible in a single volume to deal at length with more than a few topics, it is intended that this volume will be followed by others at regular intervals. In time a more complete coverage of all the varied aspects of this rapidly developing branch of science will thus be built up.

*May*, 1950

J. A. V. BUTLER
J. T. RANDALL

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# 1

# REACTION PATTERNS OF A COLIFORM ORGANISM

*A. C. R. Dean and C. N. Hinshelwood*

## 1. Introduction

The scope of this account will be defined by two major considerations. On the one hand, it will consist largely (though not exclusively) in as comprehensive a study as possible of the biophysics of a typical coliform bacterium, *Bact. lactis aerogenes* (*Aerobacter aerogenes*), and some aspects will be connected simply by their relevance to the coherence of that picture. On the other hand, it will be largely dominated by the conception of the cell as a system to which the laws of chemical kinetics, suitably formulated, can be usefully applied.

The kinetic study of cells does not mean merely the measurement of growth rates or the finding of equations expressing the variation with time of total or viable population: it is the search for the way in which characteristic biological phenomena emerge from sets of chemical reactions individually similar to those of the inanimate world, but occurring in special combinations and relations. The whole subject will be best introduced by some general observations on this idea.

Living cells can build up, from inorganic and the very simplest of organic compounds, all the complex proteins, nucleic acids, polysaccharides and other substances of which they are composed. Many of the synthetic reactions by which this is done would be attended by great decreases of entropy were they not compensated by others (such as the oxidation of carbon compounds) in which the change of entropy is an even greater increase. The linking of reactions is thus a thermodynamic necessity for cell growth.

The synthesis of complex and varied products from very simple ones demands long sequences of reactions, such sequences branching where one intermediate is used for two purposes, and converging where two separately formed intermediates have to be combined. The multitude and variety of reactions brought about by bacterial cells and the subtle variations and gradations in observed behaviour bear witness not only to the numerous elementary steps in these sequences, but also to the existence of many alternative combinations of routes to the final complex constituents. The complete network of degradative and synthetic processes, with its sequences, bifurcations and alternatives may be termed the system of *reaction patterns* available to the cell.

The raw materials available in many nutrient media are not of any high degree of reactivity in their normal states, and their utilization in the production lines of the cell manufactory will demand the intervention of intermediates of a more active character, a situation familiar throughout chemical kinetics. One of the commonest ways in which the reaction of comparatively inert substances is maintained is by the operation of catalytic cycles in which active substances are regularly regenerated, and indeed in cell metabolism cyclic combinations of reactions are almost certain to be included in the total of the reaction patterns, one of them being exemplified by the well-known Krebs tricarboxylic acid cycle, the significance of which for bacteria is discussed in section 7.

So far the reaction pattern has been thought of as an organization in time, but the cell is also spatially organized, and the interplay of the two sets of relations gives rise to some of its most characteristic properties.

The spatial organization is inherent in the very existence of the cellular structure of living matter. Raw material from the surrounding medium enters the cell by diffusion, and metabolic products leave in the inverse manner. Inward and outward diffusion being proportional to surface area, and total rate of chemical reaction to volume, the concentrations of substrates tend to fall and those of products to rise as the cell grows. Approximately constant conditions are only preserved by cell division, which normally occurs when certain key substances have been formed in more or less critical amounts. In this connexion deoxyribose nucleic acid is probably of great importance (section 11). Although a nearly steady régime is generally maintained, division is in some degree independent of actual increase in substance, so that changes in morphology may occur in suitable circumstances. (Some of the consequences of this conception are examined in section 14.) The relation of the cell to the medium imposes a system of concentration gradients throughout the system, and one enzyme in a cell may interfere with another not merely by virtue of a superior affinity for a given substrate or intermediate, but also by being more favourably placed to intercept it in passage. VIRTANEN,[1] indeed, envisages a geometrical disposition of enzymes such that those at the surface of the cell handle primary substrates and those in order towards the interior the successive intermediates of reaction sequences.

The geometrical aspect of the matter also enters in other ways. Cell enzymes resemble heterogeneous catalysts especially in that the effective activation of substrates depends upon precisely defined molecular spacings. And it is precisely in this connexion that one important function of metallic cations necessary for cell growth is exercised. The action of the metallic ion, potassium for example, as

discussed in section 13, seems to depend upon its suitability for temporary inclusion in a definite structural pattern of enzyme and substrate.

Even more generally the structural patterns and spatial organization are essential elements in the process by which living matter is reproduced. The lowering of potential energy occurring when a disordered set of units add themselves on and conform to an existing structure is an important factor by which, just as in crystal growth, the adverse entropy factor is compensated. The analogy of autosynthesis and crystal growth must not be forgotten, but neither must it be overestimated, since individual components of living cells (and even viruses) do not reproduce themselves in isolation, and the linking and organization of reactions in the temporal respect is as essential as any geometrical condition. Just as viruses multiply not by merely consuming the substance of the cell but by availing themselves of its organized machinery, so the various parts of the cell are inextricably interdependent; and autosynthetic systems do not multiply by the separate reproduction of genes endowed with some mysterious property of self-replication, but by a mutual interplay. This conception is developed more fully in section 2.

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One mode of interplay which combines in itself both the spatial and the temporal factors is that between proteins and nucleic acids. There is a connexion between the respective rates of synthesis of these two essential classes of cell constituent, and the characteristic spacings shown by the layer-like structure of the nucleic acids probably guide the addition of successive amino acid residues to the protein chain, while the spacings of the latter exert a reciprocal action on the building up of the nucleotide piles. These matters are considered in section 11.

Even in the light of such general principles as these some important cell phenomena assume a more intelligible aspect. From the very fact that growth depends not upon a single reaction but upon a long series of consecutive reactions follows the conclusion that in general time will be required before a steady state is attained, and this time represents the well known lag phase through which bacterial cultures pass before they resume multiplication if this has been interrupted. The interruption allows the decay or escape of necessary intermediates of cell metabolism, and the diffusion of such metabolites as well as their effect in removing or shortening lag phases can be directly shown (section 3).

The distinction of steady and non-steady states in growth is extremely important. In the non-steady state the most unexpected and complex relationships may appear, a fact which is not always taken sufficiently into consideration when the functioning of particular enzyme systems or combinations is studied in conditions not conducive to complete cell reproduction.

The linkage or mutual dependence of the systems involved in autosynthesis has very important consequences. It renders possible adaptive responses, which can occur automatically in such a way as to lead from an initially poor to an optimum growth rate in a given new environment. Such responses (which are dealt with in section 4) are interesting properties of linked systems, and, to say the least, highly suggestive of the adaptation of bacteria to utilize new substrates or to become resistant to originally inhibitory drugs. Before, however, the actual adaptations of real bacteria can be identified with automatic responses to environment (even though such responses are almost inevitable properties of systems to which bacterial cells must show similarity) the alternative interpretation of selection acting upon spontaneously formed mutants has to be examined. The attempt to assess the relative importance of individual adaptations and of population adaptations depending upon shifts in the proportions of different individual types has occupied much effort and led to discussions of some subtlety (section 16).

When a steady state is once disturbed, a very long time may be needed for its restoration and the cell may appear to have lost altogether a property which it formerly possessed, but which in fact it ultimately regains. Alternative reaction patterns may be in competition with one another, and the operation of one may frustrate that of another, as is seen, for example, when the utilization of ammonium salts as sources of nitrogen inhibits that of nitrate, which in the absence of the rival substrate serves perfectly well. An analogous interpretation can be given to the well known Pasteur effect where the aerobic oxidation of the carbon source inhibits its anaerobic dismutation.

The whole dynamic conception of the cell reaction pattern presupposes the existence of the appropriate material basis which is copied in the autosynthetic process. Discontinuous changes in parts of this material substructure constitute mutations, and the nature, frequency and conditions of occurrence of these form another important chapter in the biophysics of the cell. There has been much discussion about the relative significance of mutations and dynamic adjustments in individual cell phenomena, and this matter will be briefly dealt with in section 15.

## 2. Kinetic Principles[2], [3], [4], [5]

The first important matter is that a series of systems similar to enzymes and linked together in such a way that each one produces by its catalytic actions substances required for building others will function as though each of the components were autosynthetic. Thus in the simplest example we might have

$$\mathrm{d}X/\mathrm{d}t = \alpha Y \text{ and } \mathrm{d}Y/\mathrm{d}t = \beta X.$$

Neither substance possesses any mysterious self-duplicating power, yet the steady state solutions of the equations are

$$X = X_0 e^{kt} \text{ and } Y = Y_0 e^{kt},$$

where $X_0$ and $Y_0$ are the initial amounts of the two components (taken at an arbitrary zero time in the steady state itself, as in the subculture of a bacterial strain).

This result can be shown to apply to any number of such linked systems, with linear and branching sequences, for example

$$dX_1/dt = \alpha X_2,\ dX_2/dt = \beta X_3,\ dX_3/dt = \gamma X_4,\ dX_4/dt = \delta X_1,$$

or

$$dX_1/dt = \alpha R,\ dY_1/dt = aR,\ dX_2/dt = \beta X_1,\ dY_2/dt = bY_1,$$

$$dR/dt = \gamma X_2 + cY_2,$$

and so on.

In the steady state all such systems increase according to the simple autocatalytic law, and are thus effectively autosynthetic. Eventually, moreover, the amounts of the components assume constant ratios which do not change further with time.

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It is clear that the increase in substance of a living cell is closely linked with the working of its enzymes. This applies to the substance of each individual enzyme whose own reproduction is thus in the sense of the above equations effectively related to its own working. This might be called a functional theory of enzyme reproduction. The objections to it which have been expressed are based upon an incorrect interpretation of it, namely one implying that a single enzyme could reproduce itself while working in the non-steady condition of an isolated preparation. The behaviour of enzymes in such preparations cannot be a complete guide to their functioning in the co-ordinated systems of cells, where alone they are reproduced. When, therefore, we write the equation $dX/dt = kX$ for the rate of increase in the amount of an enzyme in a cell, it does not imply the power of autonomous multiplication. The process of "enzyme induction" discussed by MONOD and his colleagues[6], [7] itself implies the functioning of systems which are generally similar to those referred to above.

If cell growth is governed by equations of the sort mentioned, it follows that in a given medium the ratios of the amounts of the various constituents will assume constant values as growth continues, but that if the medium is changed in such a way as to modify any of the rate constants, these ratios change to another stable set different from the first.

The kinetic treatment of cell growth can be carried forward fruitfully without any detailed hypothesis about the precise mechanism of cell division, but one general consideration is profitably introduced from

the start. If the division process is assumed to occur when some constituent of the cell reaches a critical amount (or, what proves to be equivalent, when the concentration of something within the cell reaches a critical level) then we can write simply $Y/n = \beta$, where $n$ is the number of cells, $Y$ is the *total* amount in all the cells of the key substance and $\beta$ is a constant. With the aid of this simple device the equations can be made to take account of cell number as well as ratios of components, and there then follows by an easy derivation a further kinetic principle of great importance, namely that the proportions of constituents tends towards that which gives a maximum rate of cell multiplication in the given environment.

### 3. Non-steady States

The general principle of the non-steady state is illustrated by the simplest of the above sets of equations,

$$\mathrm{d}X/\mathrm{d}t = \alpha Y,\ \mathrm{d}Y/\mathrm{d}t = \beta X$$

The solutions are

$$X = \frac{1}{2}\left(X_0 + \frac{\alpha}{k}Y_0\right)e^{kt} + \frac{1}{2}\left(X_0 - \frac{\alpha}{k}Y_0\right)e^{-kt}$$

$$Y = \frac{1}{2}\left(Y_0 + \frac{k}{\alpha}X_0\right)e^{kt} + \frac{1}{2}\left(Y_0 - \frac{k}{\alpha}X_0\right)e^{-kt}$$

where $$\alpha\beta = k^2.$$

Suppose when $t = 0$, $Y = 0$ and $X = X_0$, then

$$X = \tfrac{1}{2}X_0(e^{kt} + e^{-kt}).$$

When $t = 0$, $(\mathrm{d}X/\mathrm{d}t)_0 = \tfrac{1}{2}X_0(ke^{kt} - ke^{-kt}) = 0$.

The initial rate of increase of $X$ is zero, that is to say, its reproduction lags. The longer and more complex the sequence of processes, the longer the lag, as may be easily shown. In certain circumstances where two substances, each of which depends for its production upon the other, start at very low concentrations the lag may become almost indefinitely prolonged.

The law of increase followed by the individual components of the system in the non-steady state is in general very complex, and particular rates of increase may even wax and wane periodically for some time (as is shown in very simple examples by the presence of sine and cosine terms in the solutions of the equations).

Diffusible intermediates play a considerable part in the establishment of the steady state and in the linking of the various enzyme reactions. When a culture of *Bact. lactis aerogenes* is approaching the end of its lag phase in a glucose-ammonium sulphate medium aspartic

and glutamic acids escape into the solution in small quantities,[8] being formed at this stage more rapidly than they are consumed in further synthetic reactions. Filtrates from grown cultures shorten or remove the lag which is shown by these bacteria when very small inocula are transferred to fresh samples of unused medium. The lag in such circumstances becomes a function of the number of cells transferred, in a way which can be accounted for by the assumption that each cell contributes to the concentration in the medium of a diffusible metabolite or intermediate utilizable by all the others.[9]

The final attainment of the steady state of logarithmic growth, where each component increases uniformly in accordance with the exponential law, demands the fulfilment of several conditions. The various diffusible intermediates must have reached their appropriate concentrations within the cells, the enzymes and the other structural elements must have increased in amount until they are present in the stable ratios, and the mechanism of division must be operating so as to maintain approximately uniform internal circumstances. These various conditions are not necessarily all reached simultaneously, or even in a regular order, and correspondingly several different types of phenomenon are manifested. Some of the major types may be classified as follows, though the distinction between the groups is not absolute.

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(1) The enzyme systems are more or less intact but the intermediate metabolites have not accumulated to the steady concentrations. The result is a lag followed by normal logarithmic growth.

(2) The enzyme systems are not intact, and fresh synthesis is necessary before full functioning of the cell is possible. According to whether the key substances necessary for division are available or not before other functions are fully restored, multiplication after a lag phase sets in either at a rate less than the optimum or with the normal rate constant. Sometimes, indeed, division mechanisms operate when general increase in substance is not yet (or is no longer) possible and a diminution of cell size then occurs.

All these phenomena are observed with *Bact. lactis aerogenes* transferred from one medium to a fresh sample of the same. The commonest causes of departure from an established stationary state are loss of intermediates by diffusion, and enzyme inactivation by ageing.

(3) The cells are transferred to an unfamiliar medium, in which a completely new set of ratios between the components has to be reached and new intermediates may come into play. Here, in general, the process of adjustment is more profound, although not absolutely different in principle from that undergone by aged cells transferred to a fresh supply of a familiar medium. In these cases there is usually a long initial lag before any cell multiplication becomes possible at all. Even when this delay is over, the growth is very irregular, showing

(e.g. in the case of *Bact. coli mutabile* transferred for the first time to a lactose medium) extraordinary arrests alternating with bursts of fresh growth as the supply of one after another of the intermediates is interrupted or resumed as a result of the unbalanced increase of the various enzyme systems. After one such long lag there is rarely need for another, provided that the transfer to further samples of the same new medium is made before there has been ageing of the cells. But the growth seldom attains its optimum rate before a good many generations of cell division have occurred. Then, eventually, a balanced state is reached and the growth curve assumes the standard logarithmic form.

The phenomena shown by *Bact. coli mutabile* on transfer to a lactose medium, like those shown by *Bact. lactis aerogenes* on transfer to D-arabinose, indicate the need for a profound re-organization of the reaction pattern and of the proportions of cell components. These deep-seated changes are sometimes thought to necessitate a discontinuous mutation of basic cell structures, but they do not necessarily do so, though they may in individual examples involve this type of modification also.

The adaptation or "induction" of a given isolated enzyme function is, in comparison with the establishment of a complete cell multiplication system, a relatively simple matter (though not without its own complications). Many observations have been made by MONOD and others[6], [7] on the development of $\beta$-galactosidase activity in a strain of *Bact. coli*. The process occurs quite quickly when cells which have once had the enzyme developed are introduced into lactose media, and the activity is also quickly lost. This, however, is only one function, while cell multiplication demands the harmonious co-operation of many functions. Some confusion between the adaptation of isolated enzyme systems and adaptation for growth with lactose as sole carbon source seems to have attended the discussion of this matter in the literature.

Once the long lag preceding growth has been traversed the cell is by some writers called adapted. But whatever terms are used it is important to realize that the major step in the acclimatization of cells to new media (preliminary mutations will be considered separately) is the establishment by growth and function (see above), of a new pattern such that the original long lag need not be traversed again. In general this is not achieved until after considerable multiplication in the new conditions has taken place.

The lag accompanying adaptation to a new source of nutrients, on the one hand, and that accompanying the simple ageing of the enzyme systems, on the other, are themselves interdependent. Sometimes a bacterial strain will grow with a long lag if transferred from active growth in a familiar medium into a new medium, but if it is allowed to

age enough to develop even a comparatively short lag in the original medium will never grow at all in the new (e.g. *Bact. lactis aerogenes* on transfer from glucose to D-arabinose).

Another form of behaviour entirely consistent with the above systems of growth equations is where a culture in active growth in one medium will grow slowly but without much initial lag in a new medium, developing a long lag in this latter as soon as it begins to age even slightly in the normal one. Combinations and variants of these types of behaviour are to be expected, and are encountered.

We now pass to the consideration of an example of an automatic adjustment in further illustration of the kinetic principles.

## 4. Illustration of an Automatic Adjustment

This section will serve to illustrate several of the principles outlined above, although it deals with a particular example. Suppose there are two enzyme systems related in the general sequence by the fact that the first, I, produces a diffusible substance, concentration $c$, which is brought into reaction by the second, II. The amounts of I and II at time $t$ are $X_1$ and $X_2$ respectively, and the cell number is $n$, so that the amounts per cell are $X_1/n$ and $X_2/n$. In a very short time from the start $c$ will reach a steady value determined by the balance between formation, utilization and loss by diffusion or other processes not useful to the synthesis. In the stationary state

$$\mathrm{d}c/\mathrm{d}t = A(X_1/n) - B(X_2/n)c - Cc = 0 \qquad \ldots.(1)$$

where $A$, $B$ and $C$ are constants. We also have

$$\mathrm{d}X_1/\mathrm{d}t = k_1X_1 \qquad \ldots.(2)$$

and

$$\mathrm{d}X_2/\mathrm{d}t = k_2X_2c \qquad \ldots.(3)$$

According to the principle that division occurs when some key substance reaches a critical amount, $n$ will be proportional to one major component of the system, say to $X_2$, so that $n = \beta X_2$, $\beta$ being a constant. Insertion of this relation into (1) gives

$$c = \alpha X_1/X_2$$

where $\alpha$ is a fresh constant.

Thus (3) becomes $\mathrm{d}X_2/\mathrm{d}t = k_2\alpha X_1$.

If $X_1/X_2 = v$, we have

$$\frac{\mathrm{d}v}{\mathrm{d}t} = \frac{X_2\dfrac{\mathrm{d}X_1}{\mathrm{d}t} - X_1\dfrac{\mathrm{d}X_2}{\mathrm{d}t}}{X_2^2} = v(k_1 - k_2\alpha v) \qquad \ldots.(4)$$

If $k_2\alpha v$ is initially smaller than $k_1$ then $\mathrm{d}v/\mathrm{d}t$ is positive and the ratio

$X_1/X_2$ increases with time. As it does so, however, $dv/dt$ according to (4) itself falls towards zero and when it once reaches this value no further change occurs. The ratio $X_1/X_2$ is now stable and $k_2\alpha v = k_1$. Samples of the system transferred to fresh medium of the same constitution continue to increase with preservation of this ratio.

But conditions may be changed so that the concentration, $c$, is reduced. For example, a toxic substance may be added to the medium and may destroy the metabolite or interfere with its formation. Lowering of $c$ means lowering of $\alpha$ to a value $\alpha'$, so that now $dv/dt$ becomes positive once more, and $X_1/X_2$ therefore increases until $k_2\alpha' v$ again balances $k_1$. When the toxic substance is first added $(1/n)dn/dt$ falls below $k_1$, but when $X_1/X_2$ rises again to the new steady value it regains its original magnitude.

This example applies particularly to a possible mode of adaptation to drugs and antibiotics, but the general principles apply to similar calculations about the automatic attainment of an optimum growth rate when inocula of a culture are transferred to a new nutrient medium.

## 5. Alternative Reaction Patterns

The systems of consecutive reactions with their points of branching, cyclical interdependences and so on which make up the reaction pattern of the cell can in principle be multiple. More than one combination is usually possible whereby a numerous and complex set of relatively simple steps can lead to given results. The state of affairs might be likened to a railway network in which various sections of track, some normally used much more than others, can be combined in different ways to give a route between defined termini.

The mathematical examination of simple models of such systems shows that where such alternatives exist that one will be selected which gives the optimum rate of growth. There is, however, an important qualification to this statement, namely that the establishment of this optimum may occur only after a long time. It may even be completely inhibited for a considerable period by the operation of a less effective but already working alternative process.

Adaptation of bacterial cultures to new substrates may depend upon the establishment of alternative reaction patterns, and there may be more than one of these possible even for a given medium. If certain basic structural elements are entirely absent from the start, then a mutation may have to precede the establishment of the new reaction pattern, but the latter process is always necessary (compare section 15).

The simplest form of the mathematical theory predicts a rapid setting up of a new reaction pattern in changed conditions of growth and a rapid reversion to the old if the cells are returned to the original environment. There are, however, reasons why on occasion the process

of adjustment may be extremely slow—though theory and experiment combine to show that eventually it will go to completion or nearly so. The fact that slow adjustment, even so slow as to simulate stability, is not evidence of a really permanent structural change is of great importance in the discussion of theories of adaptative and mutational changes. An example of the complex factors determining rate of reversion or adjustment will therefore be given in the following lines, in illustration of the kind of effects which may be expected (and are found).

Suppose there are two components present in total amounts $X$ and $Y$ respectively, belonging to competing reaction sequences and distributed among $n$ cells. Let $Y$ for its participation require a certain intermediate (concentration $c$ in cell) which is destroyed by $X$ in some enzyme reaction without useful result, at any rate as far as growth is concerned. The intermediate attains a stationary concentration given by the equation

$$dc/dt = A - Bc - CcX/n = 0$$

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where $A$, $B$ and $C$ are constants. $A$ is the rate of formation in any given cell, $Bc$ the rate of loss from the cell by diffusion, and $CcX/n$ the rate of destruction by $X$, present in each cell in amount $X/n$.

$$c = \frac{A/B}{1 + (C/B)(X/n)}$$

$$dY/dt = kYc = k_y Y/\{1 + \gamma X/n\}$$

where $k_y$ and $\gamma$ are new constants.

$$dX/dt = k_x X.$$

It follows that

$$\frac{1}{(Y/X)} \frac{d(Y/X)}{dt} = \frac{d \ln (Y/X)}{dt} = \frac{k_y}{1 + \gamma X/n} - k_x$$

Suppose when $t = 0$, $k_x > k_y/(1 + \gamma X_0/n)$, as it would be if $X_0/n$ were large, then $Y/X$ decreases without limit, and no effective establishment of the $Y$ route is possible. Suppose, however, that when $t = 0$ it happens that $k_y/(1 + \gamma X_0/n) > k_x$, as it may be if $X_0/n$ is small. Then the ratio $Y/X$ begins to increase. What then happens depends upon the condition of cell division. If $X$ and $Y$ are parts of possible metabolic routes, we shall have $X = \beta n$ or $Y = \beta' n$ according to the principle already explained. If $X_0$ is small enough for $Y/n$ to reach $\beta'$ before $X/n$ reaches $\beta$, division occurs and $k_y/(1 + \gamma X/n)$ is now equal to $k_y/\{1 + \beta'\gamma(X/Y)\}$ so that

$$\frac{d \ln (Y/X)}{dt} = \frac{k_y}{1 + \beta'\gamma(X/Y)} - k_x.$$

If this last expression is positive at the time of the first division $Y/X$ increases without limit and the $Y$ route wins the competition.

Thus we have the interesting state of affairs that the final result is determined by the initial value $X_0/Y_0$. The conditions may be such that the $Y$ route is the one corresponding to faster growth, and yet if the $X$ route is well established it can persist and inhibit the development of its rival. When, however, it once decays, by the ageing of the cells or other means, the $Y$ route may come into its own as soon as conditions favour growth once more and thereafter persist.

If $k_x$ and $k_y/\{1 + \gamma(X_0/n)\}$ are nearly equal and if the ageing of the various enzyme systems is allowed to occur in periods when growth is interrupted, an extraordinarily unstable state of affairs may result, in which there is an erratic alternation between the two growth patterns according to the immediate past history (see section 17).

## 6. Outline of Some Special Problems to be Considered in Relation to *Bact. lactis aerogenes* and Closely Related Organisms

As a typical example of one sort of reaction sequence involved in cell growth we shall consider the significance of the "tricarboxylic acid cycle," and in connexion with protein synthesis the influence of amino acids upon growth rate. Another kind of kinetic and catalytic influence will be studied in relation to the action of potassium ions in growth and cell function, and the role of iron and magnesium will be briefly touched on.

The interaction of nucleic acids and proteins will be dealt with and the idea of a key substance determining cell division further examined.

Adjustments of the reaction pattern will be studied first in relation to aerobic and anaerobic growth, the Pasteur effect, and the use of nitrate or ammonia as nutrients.

Adaptations to various substrates and the development of drug resistance will further exemplify these matters and will lead to some discussion of mutations and of population changes depending upon selection.

The balance of growth and division and its influence on morphology will be briefly touched upon, and finally the decline of the cell economy leading to the death of the cell will be dealt with.

## 7. The Tricarboxylic Acid Cycle

The tricarboxylic acid cycle[10], [11], [12], [13] constitutes part of a reaction pattern concerned with one aspect of carbohydrate metabolism, the oxidation of pyruvic acid. It is of considerable interest since pyruvic acid is a metabolite of major importance. The cycle consists essentially in an initial condensation followed by a series of dehydrogenations

and decarboxylations. In its course pyruvic acid is completely oxidized to carbon dioxide and water. Various intermediates of the catalytic cycle are themselves suitable starting materials for the formation by amination of important protein building blocks.

Evidence for the participation of the tricarboxylic acid cycle in *Bact. lactis aerogenes* has been reported by AJL and WONG[14] and by BASKETT and HINSHELWOOD.[15] AJL and WONG showed that when resting cells of this organism which had been cultivated on citrate as sole carbon source were allowed to oxidize simultaneously $^{14}C$-labelled acetate and various unlabelled intermediates of the tricarboxylic acid cycle, in turn, all of them including citrate and α-ketoglutarate became radioactive, the labelled atoms being more or less uniformly distributed among all the carbon compounds. Other experiments showed the interconversion of the cycle intermediates, and also that there was little or no dilution when labelled acetate was oxidized in absence of carrier.

BASKETT and HINSHELWOOD, with a strain of *Bact. lactis aerogenes* which had been adapted to a synthetic medium containing glucose as carbon source, found that all the tricarboxylic cycle intermediates investigated, i.e. pyruvate, acetate, citrate, cis-aconitate, α-ketoglutarate, succinate, fumarate and malate could serve as sole sources of carbon.

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These findings confirm that the tricarboxylic acid cycle is a possible route for the decomposition of pyruvate by this organism, but there is evidence which puts limits on its significance and indicates the existence of alternative reaction patterns in the metabolism of some at least of the tricarboxylic acid cycle intermediates. For example, glucose-adapted *Bact. lactis aerogenes* requires no adaptation for growth in pyruvate, citrate, cis-aconitate and malate, but with acetate, α-ketoglutarate, succinate and fumarate as carbon sources there is a long lag and a slow rate of growth at the first subculture. "Training" by serial subculture reduces the lag and leads to an improvement in the growth rate. According to the principle of simultaneous adaptation[16] no adaptation to these intermediates should be necessary if glucose is dissimilated via the tricarboxylic acid cycle. Adaptation to an earlier member of the series: acetate, succinate and fumarate, was however, found to entail adaptation to a later member but not *vice versa*, a fact which gives a direct indication of the order in which these substances are used.[15]

In their experiments with $^{14}C$-acetate, AJL and WONG have shown that, although labelling occurred in all the tricarboxylic acid cycle intermediates, the specific activity of the respiratory carbon dioxide was much higher than that of any carbon atom of citrate, α-ketoglutarate or succinate.[14] This may indicate the existence of another

mechanism for acetate oxidation, not involving the above compounds, or that the added intermediates were not in equilibrium with the intracellular substances. Further investigation however showed that when $^{14}C$-acetate was oxidized in the presence of tricarboxylic acid cycle intermediates there was dilution of the acetate. If the cycle operates there should be no dilution of acetate, i.e. no formation of acetate when di- and tri-carboxylic acids are oxidized. AJL and WONG suggest that a $C_2 - C_2$ or some other type of condensation may co-exist with the tricarboxylic acid cycle in the oxidation of acetate by citrate-grown *Bact. lactis aerogenes*. Acetate-grown *Bact. lactis aerogenes*, on the other hand, they suggest, appears to oxidize acetate by a different mechanism, that proposed being an oxidative coupling of acetate with itself to form succinate followed by a cycle involving re-forming of acetate via the $C_4$-dicarboxylic acids and pyruvate. BASKETT and HINSHELWOOD have also suggested that the combination of two molecules of acetic acid to form succinic acid would provide a possible explanation of the cross-training relationships mentioned earlier for the series: acetate, succinate and fumarate. The different behaviour of acetate-grown and citrate-grown *Bact. lactis aerogenes* in the oxidation of acetate by resting cells is evidence for the existence of alternative reaction pathways.

The investigations of DAGLEY *et al.*[17] indicate further limits for the significance of the tricarboxylic acid cycle in *Bact. lactis aerogenes*. They show that although some of the tricarboxylic acid cycle compounds and related amino acids accelerated growth, others did not, and that fluoroacetate, which has been reported to block the cycle,[18] had no effect on growth. DAGLEY *et al.* also find[19] that the ability of *Bact. lactis aerogenes* to produce pyruvic acid from various substrates is an indication of the ease with which it grows on them, for example, the accumulation of pyruvic acid during logarithmic growth in the presence of the substrates: glucose, malate, lactate, citrate, fumarate, succinate and acetate, was greatest with glucose and malate and least with acetate. Moreover, with aerated washed suspensions of cells trained to glucose, malate, fumarate and succinate respectively, the rates of production of pyruvate for various substrates were in the order: malate > fumarate > succinate > acetate, and only cells trained to acetate produced pyruvate from acetate as rapidly as from succinate and fumarate. In addition washed suspensions of acetate-trained cells gave a steady production of pyruvate immediately on aeration. No pyruvate was produced in phosphate buffer (no carbon source) and hence no significant amount of oxalacetate, its precursor in the cycle, can have been present. Fumarate-trained cells, although able to oxidize succinate, fumarate or acetate at comparable rates, had great difficulty in producing pyruvate from acetate, but did so

from the other two substrates. These results, the authors maintain, are incompatible with the tricarboxylic acid cycle. They propose a mechanism by which the substrate is oxidized via pyruvate and acetate by a non-cyclic process and acetate is converted to pyruvate by an independent mechanism which is only fully developed by training to acetate.

To sum up it can be said that the tricarboxylic acid cycle does indicate one significant pattern in the metabolism of *Bact. lactis aerogenes* in so far as the intermediates of this cycle can act as sole sources of carbon. Alternative metabolic routes do, however, exist and this imposes limits on its significance. For example, in the metabolism of glucose by glucose-adapted cells, large stretches of the cycle do not appear to be utilized to a great extent, the metabolism of acetate by acetate-trained cells seems to proceed by way of the di-carboxylic acid cycle and the oxidation of acetate by citrate-grown cells seems to require a modified tricarboxylic acid cycle or both cycles operating simultaneously.

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The state of affairs thus revealed by detailed biochemical evidence corresponds to expectations based upon general kinetic arguments. It is important for the understanding of certain surprising phenomena observed in the study of growth rates. When *Bact. lactis aerogenes* is trained to acetate the adapted strain may or may not, according to very delicately balanced conditions, show its optimum growth rate in a glucose medium. Moreover, as the cells which show impaired growth are readapted to the glucose medium they pass temporarily through an unstable phase in which marked fluctuations in growth rate occur between one subculture and the next. The fluctuations are too rapid to be explained by selection of separate mutant strains, but depend upon a state where two alternative reaction routes within the cell itself are in competition. One of the possible routes in acetate is compatible with optimum rate of growth in glucose, the other not.[20] Analogous observations have been made with other media. The fluctuations and instability of the partially adapted state can be accounted for by mathematical arguments.[3], [4], [5]

## 8. Oxidation-Reduction Relationships

Ajl and Wong[14] have reported that resting cells of *Bact. lactis aerogenes* which had previously been grown on a citrate medium were capable of oxidizing acetate rapidly whilst acetate-grown cells did not oxidize citrate significantly in 30 minutes. Experiments carried out with growing cultures[21] yielded the following results (Table 1):

When first inoculated into acetate medium glucose-trained cells have a lag of from 5–24 hours and thereafter growth is slow. Subculture in citrate has eliminated this lag and the rate of growth in acetate

TABLE 1

| *Medium to which cells were trained* | *M.g.t. (min.)* | | $\Delta L$ *(hr.)* |
|---|---|---|---|
| | *Citrate* | *Acetate* | |
| Citrate (3 subcultures) | 110 | 105 | 0 |
| Acetate (34 subcultures) | 60 | 81 | 2·9 |
| Acetate (562 subcultures) | 129 | 62 | 0·7 |
| Glucose control | 47 | 200 | 6·0* |

* Lag in acetate—lag in citrate.
$\Delta L$ Lag in new substrate—lag in substrate to which cells were trained.

of these citrate-grown cells is good. This is in agreement with the oxidation experiments of AJL and WONG. The behaviour of the two acetate-trained strains in citrate is similar to that found by BASKETT and HINSHELWOOD[15] for glucose-adapted cells in citrate. They found two types of growth, one characterized by a long lag followed by a fast rate of growth, and the second by a short lag and a slower rate of growth. Thus the behaviour of the acetate-trained cells in citrate appears to be practically normal. The apparent discrepancy between these results and those of the oxidation experiments of AJL and WONG may be due either to the fact that in the latter the tests lasted only 30 minutes, so that any lag phase of the resting cells may not have been overcome, or to the insufficient training of the acetate-grown cells. It is known that considerable training to acetate is necessary before it is utilized readily.[15], [17], [20]

In any case enzyme tests (dehydrogenase activity, oxygen uptake, etc.) need not yield the same results as growth rate measurements since in the former only partial systems are investigated whilst growth involves the mobilization of a complete set of co-ordinated metabolic routes.

Nevertheless much information has been obtained by these techniques. An example is the investigation of the reducing power of *Bact. lactis aerogenes*.[22]

The reducing power, $R$, of a bacterial culture may be conventionally defined in terms of the time taken by unit mass of cells to reduce a given amount of dissolved oxygen together with a given amount of methylene blue added as an indicator. (Cultures of *Bact. lactis aerogenes* grown in media containing different carbon sources but with growth limited by the same concentration of ammonium sulphate reach almost identical *turbidimetric counts*, which have been shown to be proportional to cell mass, although the *haemocytometer cell counts* may vary several-fold. Hence $R$ as defined above measures the activity of the culture per unit of bacterial nitrogen content.)

During the logarithmic growth phase, cultures of *Bact. lactis aerogenes* in a glucose medium possess a constant characteristic reducing power.[23] BASKETT and HINSHELWOOD[22] found that for cultures fully adapted to most of the possible carbon sources there was a basic value of the reducing power $R$ which varied only between 12·5 and 15 units, although the mean generation times ranged from 23 to 90 minutes. (With a few of the carbon sources the values of $R$ were abnormal at high concentrations of substrate but became normal in the low concentration range.) This standard value for $R$ with fully adapted cultures implies a nearly constant rate of oxygen uptake per unit bacterial nitrogen content and suggests that the optimum growth rate attainable by the adaptation of *Bact. lactis aerogenes* to a given carbon source is fixed by a rate-limiting step in the respiratory system of the cell.

During the adaptation of cells to a new carbon source, however, the growth rate and reducing power increase in parallel to their respective optima. This is easily understood when it is considered that in cells transferred to a new environment to which adaptation is necessary the various enzymes will not be present in the proportions necessary for optimum utilization of the new substrate. The oxidative mechanisms will therefore not be fully utilizable at first and the extent to which the enzyme proportions are re-adjusted will determine both the growth rate and the degree to which the oxidizing mechanisms are being used. This parallel behaviour will continue until the oxygen uptake has reached its maximum, i.e. the substrate is being oxidized at the optimum rate possible under the circumstances and at this stage the growth rate can increase no more. Since different amounts of oxygen are required for the conversion of various substrates into cell material it is quite understandable that when a constant rate of oxygen uptake is the limiting factor the growth rates of fully adapted cultures should differ widely from substrate to substrate.

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### 9. The Transition from Aerobic to Anaerobic Growth and the Pasteur Effect in *Bact. lactis aerogenes*

The Pasteur effect, a name now applied to the phenomenon in which the presence of air reduces the rate of alcoholic fermentation[24] is of interest in connection with the kinetic study of the relationship between aerobic and anaerobic growth in *Bact. lactis aerogenes*. The best definition is: the partial inhibition of the degradation of the substrate during utilization of molecular oxygen.[25]

It is not intended in this section to review the numerous investigations and theories which have been published from time to time, since critical reviews are available of which those by LIPMANN,[26] BURKE[27] and DIXON[25] may be mentioned.

With *Bact. lactis aerogenes* in the logarithmic growth phase the consumption of glucose, citrate or pyruvate is more than twice as rapid under anaerobic conditions as under aerobic conditions, although the growth rate is less.

BASKETT and HINSHELWOOD[28] in an investigation of aerobic and anaerobic growth found that when an aerobic culture of *Bact. lactis aerogenes* growing actively in a substrate which would also support anaerobic growth (for example, glucose, glycerol, citrate and pyruvate) was made anaerobic, growth was arrested for a considerable time and then re-commenced at the slower anaerobic growth rate. During this lag phase the reducing power of the cells steadily increased. On the other hand, there was no such lag when air was passed through an actively growing anaerobic culture. This indicates that although the aerobic growth mechanisms appear to be fully mobilized in an anaerobic culture, considerable reorganization of the reaction scheme seems necessary before the cells in an aerobic culture can grow anaerobically.

*Bact. lactis aerogenes* during aerobic growth oxidizes glucose completely by a reaction sequence involving many stages at some of which hydrogen is transferred to co-enzyme systems, the reduced co-enzymes being in turn re-oxidized by molecular oxygen. When the supply of oxygen is cut off, however, a new enzyme mechanism has to be developed for the re-oxidation of the reduced hydrogen carriers, and this necessary adjustment accounts for the lag during the transition from aerobic to anaerobic growth. Apart from this a common metabolic route seems to be indicated for the anaerobic and aerobic dissimilation of glucose, as is further evidenced by the ease with which glucose-trained cells can metabolize pyruvate both aerobically and anaerobically and also by the fact that a lag was obtained only in the transition from aerobic to anaerobic growth. If different metabolic routes were involved a lag would be expected for both transitions.[28]

These considerations lead to the idea that the Pasteur effect is connected with the mode of re-oxidation of hydrogen carriers in the following manner.[28] A typical carrier system may be represented as $X$, $XH_2$ (the actual number of carrier systems involved does not affect the argument). In the course of aerobic metabolism the carbon substrate is degraded by a series of oxidations during which $n$ pairs of hydrogen atoms are transferred to $X$ and $n_1$ of them subsequently removed from $XH_2$ by other carriers ultimately linked with molecular oxygen. When the conditions become anaerobic, on the other hand, these $n_1$ pairs of hydrogen atoms must be oxidized if growth is to continue by the same metabolic route, and by some mechanism other than the use of molecular oxygen. This must necessarily result in an increased, wasteful utilization of the carbon substrate itself, and thus could account for the Pasteur effect. In the steady state of anaerobic

growth a certain ratio $XH_2/X$ will prevail and this will drop abruptly when oxygen is provided, since oxygen oxidizes $XH_2$ (or something in dynamic equilibrium with it). $XH_2$ no longer depends for its re-oxidation upon extra substrate consumption, the rate of which accordingly drops, but since it is re-oxidized effectively by the oxygen itself there is no interruption of the growth.

The reduced hydrogen carriers can, of course, also be re-oxidized by the reduction of an extraneous oxidizing agent such as nitrate. This mechanism is dealt with in the section on nitrogen metabolism.

## 10. Nitrogen Metabolism

Carbon metabolism and nitrogen metabolism are closely linked, and one is to a considerable extent a function of the other. The sequence of events which may occur in the tricarboxylic acid cycle has been indicated, and it is clear that various compounds participating in this could be aminated by ammonia to give the amino acids necessary for protein synthesis. This is one of the major requirements for growth, and indeed the enzyme activity of cells grown in nitrogen-deficient media falls progressively with the nitrogen content.[1], [29]

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*Aerogenes* or *coli* strains normally require adaptation to grow with ammonium salts as the sole source of nitrogen, and may at first show a long lag when called upon to do so. In general, amino acids may be utilized in one of two ways, either by deamination to yield ammonia and appropriate carbon fragments, or directly. This is shown by the fact that some are used more rapidly than they could yield ammonia by deamination, and in many cases optimum growth rate is not correlated directly with optimum deaminase activity of the cell towards that amino acid which is provided as a source of nitrogen in the medium. With alanine and *Bact. coli*, for example, the optimum growth rate is reached without any training of the deaminase activity, yet with glycine the growth rate and the deaminase activity of the strain are closely connected. The glycine appears to act simply as a source of ammonia while the alanine is used in a more direct way.[8]

The long lag shown by *coli* strains first obliged to rely on ammonium salts for their nitrogen and given glucose for a source of carbon seems in general to be conditioned by the need for the formation of suitable carbon compounds to be aminated by the ammonia. If these are provided the lag may be very much reduced. Thus the addition of α-ketoglutaric acid is nearly, though not quite, so effective as that of glumatic acid itself.[8] The important mechanism of transamination will not be discussed here, but reference may be made to the discussion by Krebs.[30]

Partial reaction patterns such as the tricarboxylic acid cycle (and there must be a number of such routes) usually provide most of the

carbon skeletons for amination. For protein synthesis some twenty amino acids or amino acid residues must condense, and in an order which is probably governed by geometrical factors imposed by existing structures in the cell. In a medium containing all the protein amino acids ready made the growth rate of *Bact. lactis aerogenes* is greater than it ever becomes even after the most thorough training in an ammonium salt medium or in one containing a limited selection of amino acids.[31]

The meaning of this seems to be that for the protein formation the various amino acid residues must be presented in the appropriate order to a growing chain, $—A—B—C—$, if $A$, $B$, $C$ and so on are the residues in question. The rate of arrival of any one amino acid, $A'$, from which $A$ is derived will be a function of its concentration, i.e. the rate of presentation to the growing chain will be $f'_{A'}[A']$. The average time interval between the incorporation of the predecessor of $A$ and $A$ itself will thus be given by $t_A = 1/f'_{A'}[A']$ and the time taken to build up a chain will be $\Sigma t_{A'} = \Sigma(1/f'_{A'}[A'])$.

Thus the addition to the medium of each extra structurally essential acid will cause a progressive reduction in the mean generation time towards a limit. The omission of one may cause a considerable prolongation of this time, since if, for example, $1/f'_{C'}[C']$ remains great owing to a slow and difficult synthesis of $C'$, then $\Sigma t_A$ itself remains great, however small most of the other terms in it may become.

The interplay of nitrogen metabolism with the oxidation mechanisms comes into evidence when nitrate or nitrite is used as a source of nitrogen. The utilization of these sources by *aerogenes* or *coli* strains has been studied by Stickland,[32] by Pollock[33] and by Lewis and Hinshelwood.[34] Nitrate is reduced to nitrite which is then reduced further, the relative rates of the two processes varying with the strain and with the degree of adaptation. The combination with the carbon compounds may well occur at the hydroxylamine or at the ammonia stage of reduction.[35], [36]

When ammonium salts provide the nitrogen, optimum growth requires aeration of the culture, aerobic growth being much more rapid than anaerobic growth. It develops in parallel with the dehydrogenation mechanisms up to a limit determined by the reoxidation of reduced co-enzymes, as has been described above. If a culture is in active growth in nitrate and is suddenly provided with ammonium ions, the utilization of the nitrate stops at once and is only resumed after the ammonia has all been consumed, growth meanwhile continuing at the expense of the latter. The two nitrogen sources are not used simultaneously, as one might have guessed, in the ratio of the rates at which they would be used separately. The explanation is as follows. At certain stages of cell metabolism occur reduced compounds, which

may be typified as $XH_2$, and which can be oxidized, either by oxygen, nitrate or other suitable hydrogen acceptors, to $X$, the pair, $X$, $XH_2$, constituting the system coenzyme-reduced coenzyme. The good aeration required in the ammonia system keeps the ratio $X/XH_2$ high, but when the reduction of nitrate becomes necessary for the supply of nitrogen, the ratio must fall and remain low. Thus optimum growth in ammonia is incompatible with reduction of nitrate. When ammonia is added to an aerated nitrate system, $X/XH_2$ rapidly rises and the nitrate reduction stops.

An analogous phenomenon appears if the bacteria are grown anaerobically in presence of nitrate which supplies both nitrogen and oxygen. Sudden aeration gives an abrupt cessation of growth. In the absence of oxygen $XH_2/X$ rises considerably, but sudden access of oxygen causes a catastrophic fall, so that growth is arrested until a new balance can be established.

These various effects illustrate what might be called the branching of a reaction route, where a given substance or system, in this case the system $X$, $XH_2$, suffers two alternative changes in reactions which compete with one another, and where the operation of one branch may inhibit that of the other. The observations also indicate the finite time required for the establishment of steady states, both the renewed utilization of nitrate after addition of ammonium salts, and the resumption of nitrate reduction after inhibition by oxygen being accomplished only after appreciable periods of delay.

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## 11. Phosphorus Metabolism

The study of phosphorus metabolism illustrates several important principles of cell growth.

In a simple synthetic medium in which the phosphorus is supplied as phosphate ions, the organic phosphorus content of cells of *Bact. lactis aerogenes* increases gradually during the lag phase, reaches a steady value at the beginning of the logarithmic growth phase, and declines again to its original value as the stationary phase is approached. Reduction of the phosphate content of the medium to $10^{-6}$M does not significantly alter the growth rate.[37]

About 80 per cent of this total organic phosphorus appears to be in the form of the nucleic acids, deoxyribose nucleic and ribose nucleic acids (including free pentose nucleotides), and the remainder is probably present as phospholipoid. The deoxyribose nucleic acid content of individual cells of this organism grown under a wide variety of experimental conditions, including phosphorus deficiency, remains constant, although the ribose nucleic acid content and the cell size show marked variations,[38] results which suggest that the deoxyribose nucleic acid content is a major factor determining cell division in bacteria.

This constant amount of deoxyribose nucleic acid per cell is very important in the kinetic treatment of adaptation to new substrates or to inhibitors, since it exemplifies a constant factor which is directly proportional to the number of cells during the early stages of adjustment to the new environment when the enzyme systems are not in the steady state.[5]

Since the deoxyribose nucleic acid content of the cell is nearly constant, the changes in the amount of total organic phosphorus per cell during the growth cycle must be due to variations relative to it in the remainder of the cell phosphorus. During the lag and logarithmic growth phases the total phosphorus in the cell is provided by the phosphate in the medium. At the end of the growth phase, however, a certain amount of cell division takes place after the conversion of phosphate from the medium into organic phosphate has practically ceased, the cells becoming smaller in the process. Now an increase in the number of cells necessitates an increase in the deoxyribose nucleic acid content of the culture as a whole. This must take place at the expense of the other organic phosphates and since most of the organic phosphorus of the cell, other than deoxyribose nucleic acid, is in the form of ribose nucleic acid or free pentose nucleotides it is quite probable that these are converted into deoxyribose nucleic acid at the end of the logarithmic phase.[37], [38]

These observations extend and generalize those provided by the experiments of MALMGREN and HEDEN[39] on the ultraviolet absorption of bacteria during the growth cycle, and are confirmed by the work of WADE,[40] who has shown that in *E. coli* the variations in the phosphorus content of the cells can be largely accounted for by variations in the ribose nucleic acid.

Besides these variations in the ribose nucleic acid content of the cell during the growth cycle, CALDWELL, MACKOR and HINSHELWOOD[41] have found that the ribose nucleic acid content of cultures of *Bact. lactis aerogenes*, examined at a standard bacterial count during the phase of logarithmic growth, is approximately proportional to the rate at which the cells have grown. This relationship was true for normal cells grown in synthetic media containing various carbon sources, with and without inhibitors, and in broth and also for slow-growing mutants produced by ultraviolet irradiation. It suggests that ribose nucleic acid is closely concerned in autosynthesis. Further evidence for this may be derived from the investigations of CASPERSSON.[42] WADE[40] has suggested that the relationship between growth rate and the ribose nucleic acid content of the cell is not a direct one since the growth rate becomes maximal almost immediately after inoculation whereas the increase in ribose nucleic acid takes about half a generation to reach its maximum.

These results suggest certain features in a general hypothesis of autosynthetic processes. In the synthesis of protein the nucleic acid, by a process analogous to crystallization, probably guides the order in which the various amino acids are laid down, while during the formation of nucleic acid the protein molecule controls the order in which the different nucleotide units are arranged. A co-ordination of this type has been discussed by CALDWELL and HINSHELWOOD[43] and by HINSHELWOOD,[3] and has been referred to in section 1.

It seems likely that, of the cell nucleic acids, the ribose nucleic acid plays a much larger part in autosynthetic processes than the deoxyribose nucleic acid. The constancy of the latter suggests that it is responsible for the stable hereditary characters of the cell, whilst the former is probably the seat of the variable and adaptable characters.

## 12. SULPHUR METABOLISM

The total population reached by *Bact. lactis aerogenes* in a chemically defined medium increases linearly with the sulphate ion concentration over the range 1–10 mg/l. Higher concentrations do not increase the total count further. The growth rate is not influenced by sulphate concentrations in the range studied, 3–700 mg/l.

Thiosulphate, cysteine and methionine also serve as sole sources of sulphur, the growth rate and the total count per mg of sulphur being the same as with sulphate. With cysteine the lag before growth begins is slightly longer than with the other sulphur sources, but one or two subcultures in a cysteine medium are sufficient to remove it. This lag is not observed when cysteine and sulphate are present together in optimum amounts.[21]

Experiments on *E. coli* have been reported by COWIE, BOLTON and SANDS,[44] who find that the addition of sulphite, thiosulphate and cystine or cysteine almost completely suppresses the incorporation of labelled sulphate into the cell. Homocysteine, homocystine and methionine reduce the quantity of sulphate utilized but do not entirely eliminate it. Neither homocystine, homocysteine nor methionine eliminate completely the uptake of cystine by the cell and none of the other compounds suppress the uptake of labelled methionine. These results are interpreted as suggesting that the pathway for sulphate metabolism in *E. coli* is:

$$\text{"}SO_4 \rightarrow \begin{matrix}\text{"}SO_3\\ \text{"}S_2O_3\end{matrix} \rightarrow \text{cyst(e)ine} \rightarrow \text{homocyst(e)ine} \rightarrow \text{methionine}$$

It must also be assumed that methionine and cyst(e)ine can be directly incorporated into the cell protein.

The lag which occurs with *Bact. lactis aerogenes* when cysteine is the sole source of sulphur suggests, however, that this substance is not in the direct path of sulphate metabolism. This is an example of the variations in reaction pattern which can exist even in closely related organisms.

### 13. Role of Metal Ions in Growth Processes

The only metallic ions definitely known to be required for the growth of coliform organisms are potassium, magnesium and iron, though the possibility that others may be needed to perform the functions of the so-called trace elements cannot be definitely pronounced on.

From the point of view of the present discussion potassium is of special interest in that its action illustrates a kinetic mechanism of a peculiar kind. During growth and fermentation processes large amounts of potassium are taken up by the cell, but they are subsequently discharged again and appear not to be necessary for its permanent structure, organization or viability.

Many authors [45]–[58] have dealt with the influence of alkali metals generally on the metabolism of bacteria and yeasts, and it has long been clear that potassium plays a specific part in the metabolism of sugars, and also that potassium ions and hydrions may displace one another.

With *Bact. lactis aerogenes* potassium may be replaced, though with lowered efficiency, by rubidium, but not by caesium, sodium or lithium. The size of the alkali metal ion seems thus to be of primary importance. During growth of the cell and also during the oxidation of carbon substrates in the absence of nitrogen for growth, the potassium (or rubidium) ions enter the cell and at the same time, in the early stages of growth, the positive stream which they constitute is balanced by a parallel stream of negative phosphate ions.

The alkali metal ions become bound with some form of non-diffusible phosphorylated intermediates belonging to the carbon metabolic cycle. Since the ionic size must be specifically correct and not merely small enough for penetration (the larger caesium and the smaller sodium being equally ineffectual) it seems that ion and intermediate pack together into some definitely patterned structure constituted by the reacting system on the surface of the appropriate enzymes. When the geometric relations of such a system are just right it can possess considerable stability (as shown for example by the occasional insoluble salts of alkali metal cations with highly specific organic acids).

The stability of these reacting systems provides the condition which renders possible the flow of the potassium into the cell, and this in turn allows the inflow of the oppositely charged phosphate ions. Certain cells moreover (e.g. *Staphylococcus aureus*), have been shown to

accumulate free glumatic acid when incubated with glucose and glutamic acid, the process being accompanied by an uptake of potassium ions.

In growth the phosphate is incorporated in the cell material largely as nucleic acid (and glutamic acid would also be consumed), but as growth or fermentation proceeds the potassium or rubidium ions eventually leave the cell once more. One of the factors contributing to this eventual displacement is the formation of hydrions, but the situation is more complex and subtle than that describable as a simple competition. The total bacterial population of *Bact. lactis aerogenes* which a glucose-ammonium sulphate-phosphate medium will support before growth stops is in fact nearly linearly proportional (within limits) to the potassium content of the medium, so that a competition between $K^+$ and $H^+$ for the occupation of important enzyme sites is clearly enough indicated. In this sense the alkali metal ion acts as a protection against the adverse influence of hydrions. The lowering by increased acidity of the activity of dehydrogenases is in fact partially compensated by the presence of additional potassium or rubidium ions. But the cell system is essentially a dynamic one and one of the most interesting aspects of the matter is revealed if a cell suspension actively growing or oxidizing glucose is made more acid. At increased acidity the suspensions are found to contain a greater amount of alkali metal ion at the point of maximum uptake. The result of an abrupt rise in hydrion concentration is, however, to displace alkali metal ion from cells which are not actively functioning, so it is clearly some kind of adaptive adjustment which leads to the increased uptake.

What seems to happen further illustrates the principles outlined in the earlier sections. When the hydrogen ion concentration suddenly becomes adverse a whole series of important cell reactions will be slowed down. If this slowing affects the reactions which normally consume the intermediates capable of holding alkali metal ions in stable complexes, then the concentration of such intermediates will rise. More alkali metal ion can thus be bound and will therefore tend to enter the cell. Thus more of the intermediate-alkali metal ion complex can be formed, and the total rate of its consumption rises, the decreased specific rate being balanced by the extra amount. The result is a partial, though not a complete, neutralization of the adverse *p*H effect.

Alkali metal ion adjustments, it should be observed, are only one means of response to adverse *p*H. GALE and EPPS[59] found, for example that cells grown at adverse *p*H could contain enzymes in amounts which had increased in a way compensating for their lowered specific activity. *Bact. lactis aerogenes* in fact can, given time for adjustments to occur, grow at an optimum rate over a range of hydrion concentration



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which would certainly affect adversely the activity of many of its enzymes in static measurements.

Iron is a necessary element for *Bact. lactis aerogenes* but the requirements are low and optimum growth rates are observed in most media with the traces present as impurity. For optimum activity of the catalase in washed suspensions of cells $2{\cdot}5 \times 10^{-6}$ g ion/l. $Fe^{++}$ seems to be needed.[60]

Magnesium requirements though small are definite and unless one or two mg/l are present the lag phase becomes very much prolonged.[61]

WEBB[62], [63], [64], found that in simple chemically defined media bacteria failed to grow in the absence of magnesium and that the concentration necessary for maximum growth varied according to the Gram reaction of the species, Gram-positive organisms requiring about ten times as much as Gram-negative organisms. In complex media (i.e. peptone-water) magnesium deficiency or excess inhibited cell division, whereas in simple chemically defined media growth (i.e. synthesis of cell substance) was limited. He has suggested that magnesium is involved in the synthesis of bacterial protoplasm as well as in cell division and that in simple media the synthetic reactions require the higher magnesium concentration.

## 14. DIVISION

That the cell normally divides when its content of deoxyribose nucleic acid reaches a more or less standard amount has already been mentioned, and the fact that any cell constituent should, in a given environment, play the part of a pace-maker in such a way has been shown to be important in the kinetic treatment of growth phenomena.

In different media the cells of *Bact. lactis aerogenes* attain different sizes, but the DNA content is approximately constant. During the growth cycle in a given medium the cell size varies in a more or less regular rhythm, increasing towards the end of the lag phase, and decreasing, sometimes very markedly, just before the stationary phase is entered. At this stage there seems to be a reversal of various synthetic processes and DNA continues to be synthesized at the expense of material derived from a depolymerization of RNA. This indication of the dynamic internal condition even when growth is coming to an end is of general importance, as will appear further in section 19.

The rhythm of growth and division is upset in certain circumstances, especially when the cell is exposed to environmental conditions to which it is not adapted.[65], [66], [67], [68] For example, with *Bact. lactis aerogenes* division is delayed and long filamentous cells are formed (*a*) on transfer to unaccustomed osmotic conditions, such as to a dilute glucose medium from a concentrated glucose medium, (*b*) on temperature change, and (*c*) on exposure to various specifically acting drugs

such as proflavine or meta-cresol. (Phenol does not provoke filament formation.) The distribution of sizes among these abnormal cells is a broad one, and consistent with a view that division has been delayed in a way which is terminated more or less at random (as is the crystallization, for example, of a supersaturated solution).

ROBINOW[69] has shown that in bacteria internal masses of substance susceptible of special staining, and sometimes regarded as nuclear, may occasionally divide independently of the cell, and thus give rise to "multinucleate" structures. The filaments are sometimes of this nature, but not always, and their DNA content seems to vary accordingly, being abnormally high for those cells where "internal division" has occurred without "external division."

The distinction between internal and external division seems to be of general biological importance and has been discussed by KILKENNY and HINSHELWOOD.[70] With bacteria, however, the two processes are usually in step, and when they are not it is evidence of maladjustment, and leads to abnormal morphology.

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## 15. MUTATIONS

There is a certain analogy between the copying process by which living matter is reproduced and the growth of crystals. It is valid in so far as the lower potential energy which accompanies the addition of fresh units to an ordered structure is an important factor rendering possible the expansion of this structure, but the parallel is imperfect in that it takes no account of the highly coordinated interplay of processes by which the building blocks themselves must be provided. The two aspects, fundamental structure and quantitative organization in space and time, are complementary.

A large part of crystal chemistry depends upon the fact that the growth of even the simplest crystals is seldom perfect. Faults and displacements occur which exert a profound effect upon the properties of the resulting structures. So in autosynthesis it is possible to imagine an occasional imperfect alignment or deposition of fresh units. Such a fault may tend to persist.

Periodic cell division is a necessity for the preservation of an approximately steady state, and this process also may be subject to certain irregularities, whether in the amounts of matter shared between the daughter cells, or in the mode of disruption (whatever it may be) of the shared material. Furthermore, the chemical hazards, such as encounter with quanta of radiation, to which all cells are potentially exposed, are incident upon them very unequally. Certain such hazards might cause actual chemical changes in parts of the essential structure.

For all these reasons cells may be subject to what are called mutations. In principle, a mutation may cause a change in the properties of the

cell and its progeny difficultly distinguishable from one which might have been caused by an organizational change due to adaptation, in which case the mutants would be selected on growth in an appropriate new medium.

If mutations are varied enough and frequent enough, then responses of complete populations to new environments could occur, not by the direct adaptation of individuals but by selection of spontaneously occurring mutants. The direct kind of response, however, is something which the whole kinetic theory of cell growth leads us to expect, and we are therefore unable *a priori* to say whether the one or the other kind of environmental effect is likely to be more commonly met with.

In the past the direct response has been ruled out as a Lamarckian inheritance of acquired characters, believed impossible. This negation is illogical, as is now more generally recognized. The usual bacterial adaptations are not in fact completely irreversible, though often rather persistent, and perfectly valid reasons can be given why the reversal of a purely organizational change may sometimes be very slow.

It will now be desirable first to consider the subject of mutations in themselves and then in the subsequent section the question of their importance in bacterial chemistry relative to adaptive adjustments of individual cells.

Mutational changes may be induced in bacteria by various chemical agents such as nitrogen-mustards,[71] dyestuffs,[72], [73] proflavine,[72] sodium deoxycholate[72] and manganous chloride[74] and by radiations (ultraviolet light, x-rays and $\gamma$-rays). Various types of mutants have resulted, such as those showing increased resistance to drugs or to phages and the so-called "fermentative" and "nutritional" mutants. Fermentative mutants are unable to carry out the fermentation reactions characteristic of the normal organism, whilst nutritional mutants are unable to grow in the medium in which the untreated organism grows unless it is supplemented by one or more amino acids or growth factors. This class of mutants has been extensively investigated and ingenious methods, such as the "screening" method of LEDERBERG and TATUM[75] and the penicillin method of DAVIS,[76] have been devised to facilitate their isolation.

The standards adopted by different workers when testing for "nutritional" mutants vary considerably. For example, LEDERBERG[77] describes such mutants as having reproducible, specific nutritional requirements while other workers have fixed arbitrary times at which growth is adjudged positive or negative. The question of standards has been discussed by DEAN and HINSHELWOOD.[78] In many cases the exacting strains have eventually become non-exacting and this has usually been assumed to be due to reverse mutation (see for example RYAN and SCHNEIDER[79]). Experiments which will be discussed later

will show, however, that the picture may be more complicated than this view suggests.

An interesting suggestion was made by REID[80] concerning the mutagenic action of ultraviolet light. He visualizes that the radiation causes a breaking of bonds which is propagated as a sort of chain reaction destroying or depolymerizing large tracts of the macromolecular cell substance. This destructive action explains the experimental findings that in practically all cases irradiation leads to a loss of existing functions rather than to the development of new ones. RAVIN[82] has pointed out, however, that the techniques used for the isolation of mutants in most of the published experiments have been specially designed to select deficient mutants, and would therefore allow positive mutations to pass unnoticed. The development of new functions is theoretically possible and speculations on how this could happen have been put forward by DEAN and HINSHELWOOD.[81]

It is well-known that cells damaged by ultraviolet light sometimes recover their lost properties by a process of re-training. This might involve adaptive repair of damaged but not completely eradicated systems, or it might involve reverse mutations. The process by which this recovery takes place was studied by PEACOCKE and HINSHELWOOD[83] and in more detail by DEAN and HINSHELWOOD.[78]

*Bact. lactis aerogenes* readily utilizes ammonium salts as sole nitrogen source, and the effects of ultraviolet irradiation on this property was studied. The irradiation was continued until about $10^5$ of the original $10^9$ cells per ml survived. These survivors were isolated and were compared with normal strains in respect of their ability to utilize ammonium salts. The criterion adopted was the difference, $\Delta L$, between the lags in synthetic medium containing ammonium sulphate and glucose, and a parallel medium containing also asparagine and glutamic acid. All the strains grew readily in the asparagine-glutamic acid medium, but irradiation increased progressively the proportion which showed large values of $\Delta L$. A second irradiation reinforced the effect of the first in that it resulted in still larger values of $\Delta L$. The distribution of values was in all cases more or less continuous. All the strains were capable of re-training to optimum utilization of ammonia by serial subculture in a synthetic medium containing ammonium sulphate as sole source of nitrogen.

In further experiments the irradiation was continued until between 100 and 10 bacteria per ml survived out of an original population of $10^9$ per ml. The survivors were isolated by a conventional screening method and all the strains which this technique indicated as mutants were tested for growth in a synthetic medium (in which the unirradiated strain grew readily) and for their sugar fermentation reactions. These strains showed an almost continuous spectrum of behaviour in their



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growth rates and sugar tests. Some strains were unable to grow at all in the synthetic medium but did so after one subculture in asparagine-glutamic acid medium. A similar pattern of behaviour was obtained when the radiation from $^{35}S$, proflavine and Fenton's reagent were used as mutagenic agents. On serial subculture in the synthetic medium all the strains eventually grew at the normal growth rate with normal biochemical reactions. A detailed quantitative study of the recovery of these properties led to the conclusion that an adaptive hypothesis involving the repair and expansion of damaged enzyme systems introduced fewer difficulties and assumptions than a theory involving reverse mutation.

Dean and Hinshelwood[84] found that with 1,092 strains isolated from irradiated cultures of *Bact. coli* by various techniques 78 had nutritional requirements. These requirements, however, showed varying degrees of complexity and indicated an almost complete spectrum of behaviour. For example, with some it was only necessary to supplement the synthetic medium with asparagine, others grew in asparagine-glutamic acid medium and the remainder required other amino acids. With some strains any single amino acid was sufficient, with others one of a group of two or three amino acids sufficed. One specific amino acid was necessary for other strains, while a few required a mixture of several, and one strain appeared to require a complex mixture of amino acids and growth factors.

Of these 78 strains only two proved to be stable. The others were re-trained to grow at the optimum rate in the synthetic medium by processes varying in complexity from a single subculture in the presence of the requirement to successive subcultures through progressively simpler media. Quantitative measurements showed that the recovery of the normal growth rate was gradual and continuous.

On the whole, therefore, it seems that the degree to which the cells become exacting on irradiation varies according to an almost continuous spectrum and that the pattern of recovery is one of continuous repair rather than the emergence at any stage of a qualitatively different organism.

One is inclined to agree with Ravin[82] when he says "because the geneticist has been primarily interested in genetic changes, cases of unstable, reversible modifications underlying heterogeneity have been either neglected, forgotten or unpublished."

### 16. Adaptation and Mutation

When first subcultured into new media bacteria frequently show reluctance to grow. One of the most characteristic of several types of behaviour is a long initial lag followed by growth at a rate below the optimum which is later reached after a number of subcultures.

A kinetic interpretation is that when the cell is growing in a medium to which it is adapted the enzymes are present in stable proportions compatible with optimum growth rate, and that when the environment is changed these are first upset but eventually settle down to a new equilibrium corresponding once more to the best growth rate possible.

Although an automatic response of this nature is to be expected in principle, and is consistent with many observations, some biologists have been reluctant to accept it, preferring to extend to bacteria the principles of the genetics of higher organisms and to believe that adaptation to a new environment is due to mutation and selection.

The nature of mutations has already been discussed. They are usually assumed to occur in a very few members of the population which eventually outgrow the rest. Mutations in the sense of basic structural changes of a discontinuous character, and automatic adjustments in cells both being possible in principle, the two views about the development of given properties in cultures are not incompatible with one another. It should also be pointed out that the difference between them is not quite as great as might appear, since in one case we deal with the selection of a few mutant cells and in the other with a kind of internal selection among possible reaction patterns. Since the requisite crossing experiments can rarely, if ever, be carried out, the use of the term "genetic" in connexion with mutations in bacteria is hypothetical. The primary problem is to decide what proportion of the population actually takes part in the adaptive process.

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According to the uncompromising mutation theory the mere length of the initial lag often observed itself shows that the proportion of pre-existent mutants must be minute. But if, as many authors[6], [85], [86] now assume, mutants themselves require adaptive development, then nothing can be inferred from the time relations alone. Once, however, the possibility of an adaptive process is admitted at all, then there is no reason why in particular cases all the cells should not be adaptable. A preliminary mutation may indeed be said to be necessary: but this means no more, necessarily, than that at some stage of its history the cell acquired a certain special type of structure, and this may have happened in remote ages. There is no need to suppose that it must have occurred during the negligible period of time in which the strain has been under experiment. There is thus in principle an open question in each specific example. Some typical cases will therefore be briefly reviewed.

Belief that resistance to drugs arises by spontaneous mutation has been largely based on the LURIA and DELBRÜCK fluctuation test,[87] the applicability of which has, however, been contested.[88], [89], [90], [91], Recent attempts by RYAN[85] to show that it really proves mutations, by use of the LEA and COULSON quantitative distribution, have also

been questioned.[92], [93] Experiment has shown that survival on a drug plate is at least a more complex affair than the mere selection of mutants, since in some cases the survivors themselves do not prove to be resistant on re-test, a finding which is more easily compatible with adaptive theories.[81]

The adaptation of *Bact. lactis aerogenes* to utilize D-arabinose has been extensively studied and illustrates several types of test.[94], [95], [96], [97] The utilization at a first subculture is characterized by a lag of several days. Towards the end of this there is a considerable increase in cell mass before any detectable increase in the actual number present. This indicates that the bulk of the population is indeed undergoing a change. Moreover, if samples are withdrawn at intervals during the lag and are plated on agar plates containing D-arabinose, all the cells yield colonies eventually, and the time required for them to appear decreases progressively as the period of sojourn of the parent culture in the liquid medium increases. A mutation hypothesis will not explain this. The last observation rules out the objection[85] that mutants may have arisen in microcolonies formed by initial rapid growth on impurities in the media. Furthermore, during the very early stages of growth no stably trained cells are detected, all undergoing rapid reversion if grown in absence of arabinose (and under conditions where the selection of reverse mutants or unchanged cells could not be important).

There seems to be little doubt here that the process of adjustment does occur, and that it occurs in most of the cells. A more controversial example is that of the *mutabile* strain of *Bact. coli*.[85], [93] At a first subculture in lactose this shows a long lag, so that the selection-mutation-adaptation issue arises. When small inocula are plated on lactose agar all cells eventually form colonies. *Prima facie* this indicates that all are adaptable, but RYAN[85] argues that microcolonies are first formed on impurities, and that each is large enough to be certain to contain a mutant which then develops further. DEAN and HINSHELWOOD[93] point out, however, that the time to the first appearance of the true lactose colonies is at first long but progressively shortens as the parent culture from which the plating samples are taken passes through its lag phase. This suggests continuous changes during the lag itself. RYAN bases a good deal of his argument upon a statistical analysis of the number of rapidly developing colonies derived from a large number of cultures grown from small inocula. According to the Lea-Coulson theory the distribution is characterized by the fact that mutants arising early in the history of a given culture will have multiplied largely by the time of plating, while those arising late will not. RYAN finds approximate agreement with the form predicted. But, according to DEAN and HINSHELWOOD, other assumptions may lead

to not dissimilar distributions: RYAN'S agreement is not exact, and in any case the theory postulates a one-step mutation while there is reason to believe that the mutants, if they are mutants, must arise in a whole series of steps (an assumption needed to account for the whole series of observable growth rates), so that the theory would in any case be inapplicable.

The related enzyme system, $\beta$-galactosidase, studied by MONOD and others,[6], [7] requires a period of "induction" before functioning at optimum rate. RYAN and the MONOD school, therefore, postulate adaptive processes following the assumed initial mutation. Thus in this example the issues in dispute narrow themselves essentially to quantitative ones.

In many cases of drug resistance the degree attained is quantitatively and continuously graded to conform to the concentration of the drug at which the culture has been trained.[98] When this fact is considered in the light of what has been said about reaction patterns a simple explanation can be envisaged, which in some cases at least seems preferable to the alternative assumption of a very complex polygene system.

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During adaptive changes violent fluctuations in growth rate may occur, and although the competition of reaction patterns could explain these, proportions of normal and mutant cells could hardly change at the rate needed.

Finally, much has been based upon the supposed permanence of some adaptive changes, which was at one time taken to show that any kinetic theory of them would demand the "inheritance of acquired characters." Not only has the logic of this been challenged, but the alleged permanence has been shown to amount to no more than sluggishness of reversion. This matter is dealt with more fully in the following sections.

## 17. ALTERNATIVE METABOLIC ROUTES[20], [78], [99], [100]

In some cases there are multiple metabolic routes by which growth in a given substrate or combination of substrates can take place. For a given medium there will be an optimum route giving, when it is fully developed, the maximum possible rate of growth, associated with the establishment of the appropriate quantitative proportions of the various cell components.

Sometimes it seems that competing reaction patterns persist as potential routes for given cells and that even in a given medium, one or other may function according to circumstances. When the cell economy is not in the steady state corresponding to continued logarithmic growth, a lag phase is traversed before this steady state is set up. Whenever growth is interrupted the lag tends to reappear.

Different metabolic routes are associated with different lags, and, since the relevant intermediates are lost at different speeds, the appearance of the lag on ageing, and its length when present will differ for different routes. Thus we may have a metabolic route which gives an inherently smaller growth rate functioning in competition with a potentially more effective one simply because it is attended with a shorter lag, or is, as it were, more readily mobilized than its competitor.

Considerations of this kind explain rather surprising phenomena which sometimes occur during training of bacterial strains, or when the training is being lost. Sometimes a phase of slow growth is suddenly succeeded by one of more rapid growth, the picture being repeated on subculture after a small amount of ageing, so that the transition to faster growth does not represent simply the moment at which faster-multiplying mutants attain predominance. Again, in the training of *Bact. lactis aerogenes* to acetate and other media remarkable fluctuations in growth rate occur from one subculture to the next, of a kind which could not possibly be explained by selection, and which represent the alternating dominance of one or other of competing metabolic routes, one lagging on occasion while a less effective competitor operates.

At times, training of bacteria to utilize a new source of material involves loss of perfect adaptation to the medium from which they were originally transferred. At others, the same training gives a strain still capable of optimum growth on return to the original medium. It seems that here, too, there are alternative metabolic routes in the new medium, one of which is compatible with optimum growth in the old and the other not.

## 18. The Time Scale of Adaptive Changes

The most frequently expressed doubts about the reality of adaptive adjustments[101] have been based upon the difficult reversibility of many changes which a new environment may call forth in bacteria. The question of the real degree of stability of the adaptations thus assumes importance.

According to the most elementary form of the theories outlined earlier the establishment of new enzyme proportions, and the organization of new metabolic routes should be rapid, and approach completion as soon as the material synthesized in the fresh conditions has diluted out that originally present. Adaptive changes are indeed often nearly complete after a few generations, but not always. They may be very slow, and, in particular, the reversion to something like the original state which should occur on return to the former medium may be long delayed. The persistence of the change is sometimes so marked as to have given rise to the idea of real permanence. This, however, seems to be not correct, and in general adaptive changes are reversible,

though sometimes at a surprisingly slow rate.[20] Several factors explain this relative inertia.

The first is the insensitiveness, in certain conditions, of enzyme reactions to the concentration of the soluble substrate.[102], [103] Suppose, for example, that in response to inhibition by a drug, a large expansion of a particular enzyme system occurs and results in enhanced production of an antagonizing metabolite. Now suppose the trained cells are returned to a drug-free medium. Over-production of this metabolite will occur. If the rate of its utilization increases with its concentration, then other enzymes now expand in their turn until, as calculation shows, the original ratios are restored. But suppose, as may happen, that these enzymes are already in the untrained cells saturated with the metabolite. Then in the trained cells there will be no increased rate of operation corresponding to the increased concentration. There will then be a sort of neutral equilibrium in which are maintained enzyme proportions, unstable in the sense that they would not spontaneously arise, but persistent in the sense that there is no special reason why they should rapidly change.

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A second factor is the existence of the alternative reaction routes.[4] If a less efficient route exists and allows growth to occur in competition with a more efficient route attended for the time being with a lag, then the establishment of the latter will be delayed. If the use of the first route involves an actual consumption of metabolites required in the second, then the delay may be indefinitely protracted (compare Section 5). There then follows the paradoxical result that training occurs more readily if the whole bacterial system is allowed to age in presence of the source to which training is desired. The active competition of the second best growth mechanism may thereby be eliminated, and from the new starting point the mechanism which is inherently the more efficient may come into its own.

A third possible factor is the following. Enzymes present molecular arrays with regions, not unlike surfaces, where heterogeneous reactions take place. The spacings and the distances between active groups will determine the chemical activity. In view of the complex system by which proteins are folded and nucleic acids packed in the cell there may be some element of randomness in the occurrence of particular spacings or local surface patterns, so that enzyme function may be confined to small regions, and may vary from one area to the next. Some regions of the internal surface of the cell may possess enzyme properties such that they can deal with substrate $A$, others such that they can deal with substrate $B$, and others again which can deal with both. The first stages of adaptation to $B$ of a cell already accustomed to $A$ may involve an expansion of the $B$-type sites. But more thorough adaptation may involve an expansion of $AB$-type sites. These being

as useful to the cell in the metabolism of $A$ as of $B$, will not be eliminated, in whichever of the two substrates growth occurs. Hence adaptation to $B$ is stable when it has involved expansion of $AB$ sites since these continue to be synthesized in the medium containing only $A$.[103]

The old idea that spacings are of primary importance shows how mutations in the sense of uncontrolled random variations in the texture of the cell substance might determine not so much the existence of a given function in a cell, as the capacity for response to direct adaptive influences. In the last resort the two views of adaptation and mutation merge imperceptibly one into the other.

### 19. Decline and Death of Cells

The probable depolymerization of RNA, the reversal of synthesis and the building up of DNA at the expense of the fragments has already been mentioned as an accompaniment of the end of growth. The process illustrates a fact of cardinal importance, namely that bacterial metabolism does not cease with visible growth, that internal exchanges continue, and that when certain syntheses can no longer be effected at the charge of the medium, they may occur at that of the existing material of the cell. Most enzyme processes are reversible, and indeed in ordinary chemistry such processes as polymerization and condensation give place to depolymerization and hydrolysis when conditions change. The reaction patterns of cell synthesis are complex and the products varied. The multiple enzyme processes are therefore unlikely, when growth approaches its end, to wane in activity in complete unison. One set of enzyme components, proteins, or nucleic acids may be temporarily built up at the expense of others.

The lively interchange of material in non-proliferating *Bact. lactis aerogenes* has been shown by the use of radioactive tracers to occur with sulphur,[104] phosphorus[104] and carbon compounds.[105] Exchange with the medium goes on while the cells are alive but stops abruptly when they are killed. Thus the traffic observed is the result of metabolic activity and not simply of decomposition.

The elaborate interdependence of the various enzyme functions of the cell has already been emphasized. In the period when the external sources have failed, one of the partial systems constituting the whole may begin to increase to the detriment of others, but it will not be able to continue indefinitely to do so. For presently those which supply it must become so weakened that they are no longer able to perform adequately functions upon which, directly or indirectly, the predatory system itself depends. It, in its turn, will enter into a phase of decline. But as it now suffers some degree of degradation or lysis it yields back to the internal medium of the cell material which serves

for the repair of other parts. With each part related in this way to others, the conditions for a quasi-periodic waxing and waning of various cell constituents can arise. The system will not be a truly periodic one since there are progressive and irreversible losses into the external medium, and the increases and decreases of individual functions are superposed on a general decline. In a complex system there will be no particular phase relation between the various activities and it will be a matter of chance if at a certain time several of the important ones pass through their minima more or less together.

These considerations give a simple and rational interpretation of the law determining the death rate of populations of bacteria.[110] As is well known, the number of survivors, $n$, out of an initial population $n_0$ in decline is often approximately, though seldom closely, represented by the law $n = n_0 e^{-\lambda t}$, where $\lambda$ is the death rate constant. The rough conformity with this law implies that the chance of death is nearly independent of the previous history of the cell. This has led to the idea that mortality depends upon random encounters with lethal agents which attack sensitive targets in the cell. The theory has not unnaturally invoked scepticism, and the significance of the law itself has been denied.[106], [107], [108], [109]

Matters are seen in clearer perspective in the light of what has just been said about the complex reaction pattern. All that need be assumed is that if enough different cell functions pass through their minima more or less together, the viability becomes irretrievably impaired and no further recovery is possible. The quasi-periodic nature of the various changes makes the fulfilment of this lethal condition random, and in large measure independent of the previous history. In such a case the exponential law will be roughly followed.[110], [111] Neither this independence, nor the law itself is absolute. There are clear indications of progressive processes of decline preceding the final irrevocable occurrence,[112] and also of adaptive adjustments to preserve the declining economy.[113] But these effects represent distortions of an exponential law rather than fundamental negations of it, and are in themselves to be expected in the light of the theoretical principles.

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# THE PHYSICAL CHEMISTRY OF HAEMOLYSIS AND BACTERIOLYSIS BY SURFACE ACTIVE AGENTS AND ANTIBIOTICS

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## Introduction

It is widely recognized that many cellular systems depend for their activity on the presence of surface regions of specific osmotic properties. These cell membranes are often associated with well-defined cell wall structures. Both the membranes and walls are regions in which considerable localization of enzymes can be demonstrated,[1] and these enzymes will play an important role in the transport of metabolites and other compounds into and out of the cells. The marked modification of the permeability properties of a wide range of cells by surface active agents has now been amply demonstrated. Studies have been made with red cells,[2] bacteria,[3], [74] yeasts,[4] paramoecia[5] and spermatazoa.[6] The diversity of the effects of detergents on these cells is not explicable in terms of simple surface tension effects alone. A great deal of information on the properties of cell membranes has been obtained by comparing the effects of detergents on the cells with the reactions of the detergents at air/water and oil/water interfaces with typical cell membrane and cell wall components (e.g. lipids and proteins). The success of this comparative approach is largely due to the fact that membrane and wall components can often be spread as insoluble monolayers.[7], [8] These monolayers are oriented, and can therefore be regarded as analogous to the oriented systems in the intact organism.[7], [8] The monolayer interactions of lipids with detergents are the best understood of the range of possible reaction systems, and the physico-chemical information is therefore most complete for cells with membranes containing a high proportion of lipids. Furthermore, the physico-chemical analogies to the membrane effects of surface-active agents apply best to a cell such as the erythrocyte, which is of low metabolic activity and therefore relatively uncomplicated. When bacteria are considered, it is clear that the detergent reactions at the walls and membranes are much more complex in view of the high metabolic activity, cell division, variations

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of morphology, capsulation, etc. The term "bacteriolysis" is thus of very broad connotation, and is taken here to describe leakage of cell solutes of fairly small molecular weight. The description of the effects of detergents on bacteria in terms of surface chemistry is thus less direct than for red cells. Apart from the studies on the effect of detergents on cells, the methods of surface chemistry have proved of value in elucidating the mode of action of polypeptide antibiotics. These antibiotics, compared with the detergents, are specific in their action. Useful information has been obtained, however, from the study of their interactions with typical cellular lipids.

This review does not include discussion of the haemolytic effects of protein lysins such as streptococcal haemolysins nor is the effect of lysozyme on bacterial walls discussed in detail. The review has divided itself naturally into sections following the various interests of the collaborating authors. The sections are those dealing with haemolysis (B.A.P.), polypeptide antibiotics (A.V.F.) and detergents effects on bacteria (M.R.J.S.).

## Physical Chemistry of Haemolysis by Surface Active Agents

The mammalian erythrocyte is in many ways a suitable cell for examination by surface-chemical techniques. Its metabolic activity is low.[9] The surface of the erythrocyte appears to be lipoidal, unlike the surface of leucocytes.[10] The stroma ultrastructure contains nearly all of the cell lipid,[11], [12] which consists primarily of phospholipid and unesterified cholesterol[13], [14], [15] in association with elenin,[16] [17] and other proteins and enzymes, notably cholinesterase.[18] Numerous studies have been made of the breakdown of the erythrocyte ultrastructure caused by surface active agents. Contradictory reports on detergent haemolysis have been due in part to the uncritical use of impure surface active agents. The surface properties of these compounds are markedly altered by impurities,[19], [20] and it is to be expected that haemolysis would also be affected. An outstanding example is sodium taurocholate. The pure substance was shown by Pethica and Schulman[21] to be poorly lytic. The good lysis obtained with crude bile salts is probably due to fatty acid impurities. Similarly, it is only recently that studies with saponin have been carried out by Ruyssen[22], [23] with well characterized compounds.

A number of attempts have been made to correlate the haemolytic activity of surface active agents with their surface tension properties. The simplest correlation, of surface tension with haemolysis, is only applicable to restricted groups of lysins. A simple relation is obeyed within a homologous series of fatty acids,[24] and other restricted groups, but no general correlation exists for all detergent lysins.[25], [26]

It was shown by SCHULMAN and RIDEAL[27], [7] that the haemolytic activity of a number of long chain ionic detergents can be related to their penetration into monolayers of cholesterol.[27], [7], [4] Similarly, the haemolytic properties of saponins are explicable in terms of monolayer reactions with cholesterol.[27], [24], [25] Recently, a general theory of the mode of action of surface active lysins has been put forward by PETHICA and SCHULMAN.[2] The lysis by surface active agents of human erythrocytes in neutral saline suspensions was shown by these authors to be of two types, depending on whether the activity at the cell surface could be related to either the surface tension lowering of the lysin itself, or to its ability to react with cholesterol in monolayers. The cell envelope is a structure involving a cholesterol-phospholipid-lipoprotein-complex.[2] A decrease in the general or local two-dimensional free energy (increase in interfacial pressure) of this complex is accompanied by structural modifications in some ways analogous to the changes in surface density and orientation found in spread monolayers on the Langmuir trough. In particular, the cell surface complex can be regarded as possessing a critical collapse pressure. The selective permeability and osmotic properties of the red cell appear to be the function of a complex or component of a complex that is irreversibly disordered or displaced above a critical interfacial pressure corresponding (at *p*H 7 and 20°C) to 34 dynes/cm at the air/water interface. This critical pressure is achieved in one of two ways. Either the lysin itself has the necessary surface pressure, or it causes an increase in the surface pressure in the cell membrane by its ability to react with one or more of the stroma components. In the first case, the lysin haemolyses by simple detergency and will cause the complex to collapse irreversibly at all concentrations of the lysin that give a surface pressure above 34 dynes/cm. This state of affairs was shown to hold for the non-ionic surface active lysins studied.[2] In the second case, the critical pressure is reached by the reaction with the stroma cholesterol, and the surface pressure of the lysin alone will be variable and less than 34 dynes/cm at concentrations just causing complete lysis. This behaviour is shown by the long chain ionic lysins and saponin.[2] The site of action of the non-ionic lysins cannot be deduced from non-specific quasi-thermodynamic studies of this sort, but further information on the primary site of action of the long chain ionic lysins has been obtained from a study of the energetics of monolayer interactions between these lysins and typical stroma components. Cholesterol monolayer penetration by the dodecyl ionic detergents is a process that rapidly reaches equilibrium. It has been shown that the equilibrium penetration decreases in the order

$$C_{12}H_{25}—SO_4^- > —SO_3^- > —NH_3^+ > —COO^- > —NMe_3^+$$

The same sequence is obeyed for the haemolytic activity of these compounds.[2] The previous reports[4], [27] giving the amine as the most lytic and penetrating dodecyl compound were probably in error because of the presence of longer chain impurities in the amine.

*Haemolysis by ionic detergents*

In assessing whether the penetration data is sufficient evidence for suggesting cholesterol as the primary reaction site of these ionic detergents, several factors must be taken into account. First, the

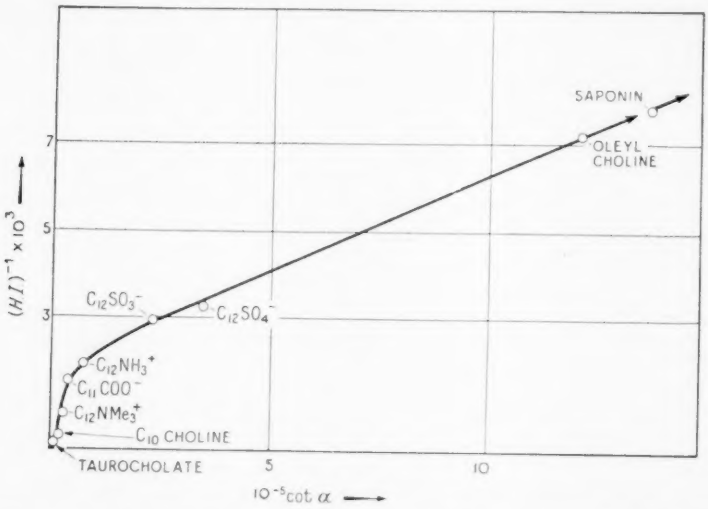


Fig. 1. The relation between haemolytic activity (H.I.$^{-1}$) and cholesterol monolayer penetration (cot $\alpha$) for a range of ionic surface active agents and saponin. The dodecyl ionic compounds are denoted $C_{12}$, and the decyl as $C_{10}$. The choice of H.I.$^{-1}$ and cot $\alpha$ to describe haemolysis and monolayer penetration respectively is discussed in Ref. [2]. Data from [2] and [28].

comparison of equilibrium monolayer data with kinetic haemolysis experiments is open to question, and the choice of a haemolytic end point is therefore important. In the experiments cited, the haemolytic end point was chosen for as slow a haemolysis rate as conveniently possible (90 minutes) without entering regions where secondary effects become important.[2] Under these conditions, comparison of the equilibrium data with the slow kinetics is quite reasonable, since the rates of the irreversible haemolytic reaction will then be proportional to the small concentration deviations from the supposed critical equilibrium state between cholesterol and the lysin.[30] In the event of the lysin not reaching a rapid equilibrium with cholesterol, comparison between monolayer processes and lysis is more complicated. The

comparison between lysis end-points and surface pressures attained in cholesterol monolayers on injecting the lysins into the subphase is quite arbitrary, and depends on the time chosen for reading the pressure rises. On the other hand, CROES and RUYSSEN[31] have obtained useful results by comparing rates of monolayer penetration with haemolysis rates for a single lysin, and varying the monolayer subphase and red cell suspension medium. In this way, some of the effects of calcium salts on saponin and digitonin lysis have been shown to be parallel to the effects of calcium ions on penetration by these agents into mixed cholesterol-lecithin films.[31] It is also of interest to observe whether the cholesterol penetration-lysis relation is obeyed by lysins other than dodecyl compounds. It has been shown that taurocholate[2] and decyl and oleyl choline iodides[28] fit the cholesterol penetration-haemolysis relation very well, as is shown on Fig. 1. All these lysins give stable penetration pressures. Dodecyl choline iodide fits the sequence of Fig. 1 only approximately as this compound does not rapidly attain stable pressures with cholesterol films.[28] Similarly, saponin does not give equilibrium films, but does fit the general sequence.[2]

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Secondly, the possibility of a reaction sequence for other stroma components in the same order as the haemolysis sequence cannot be overlooked. The possibility of either a phosphatide or cholinesterase being primary reaction sites has been examined by ANDERSON and PETHICA.[28], [29] It was shown that the reaction between dodecylamine or dodecyl sulphate with lecithin monolayers was in the opposite order to their haemolytic properties, dodecylamine penetrating more efficiently (Fig. 2). This result will hold for other possible stroma phosphatides, such as cephalin, which are less basic than lecithin itself. Red cell cholinesterase is only slightly inhibited at sub-optimal concentrations of acetyl choline in the presence of lytic doses of long chain ionic detergents.[32] It is also known that the long chain choline iodides, containing the choline head group, fit the cholesterol penetration-lysis relation, and also obey the Traube Rule for the effect on haemolysis of varying the chain length of the alkyl portion of the molecules.[28] It seems clear that phosphatides and the cholinesterase are not primary sites of detergent lysis. Their role in modifying the detergent haemolysis is discussed below. Further thermodynamic evidence for regarding cholesterol as a primary site of reaction can be obtained from comparison of the free energies of the reactions of the ionic detergents with typical proteins or monolayers of cholesterol and lecithin. The free energy ($-\Delta G^{\circ}$) of binding of dodecyl sulphate to serum albumen (as a model) in bulk is not greater than 10 Kcal.[33], [34] The free energy of penetration of the same compound into a cholesterol monolayer at about 38 $Å^2$/molecule on water is 14–16 Kcal. at a film pressure of 34 dynes/cm.[35] The comparison is by no means exact, but indicates

that this typical lysin probably would react more with cholesterol than with a protein. In comparing the free energies of dodecyl sulphate penetration into cholesterol and lecithin on isotonic saline substrate, difficulties arise in the choice of standard states for defining activities.[35], [36] For a suitable standard state, the $-\Delta G^{\circ}$ for the cholesterol reaction is greater than that for lecithin films at the same initial

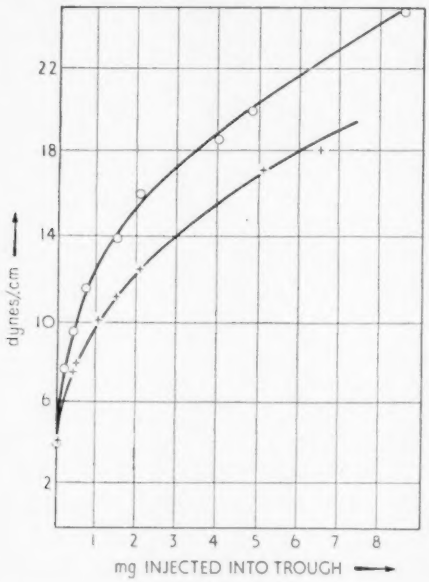


Fig. 2. The penetration of dodecylamine and dodecyl sulphate into lecithin monolayers spread on isotonic saline at *p*H 7. Trough volume 1·2 litre. ○ Dodecylamine hydrochloride. + sodium dodecyl sulphate. Ref. [28].

pressure by 2·5 Kcal. This again points to cholesterol as a primary site for reaction of the lysin.

*Ion effects and the role of stroma cholinesterase*

The action of ionic detergent and saponin lysins is modified by many factors, of which two will be considered here. The influence of polyvalent cations in accelerating haemolysis by anionic detergents[37], [38] is likely to be due to modifications of the properties of the stroma phosphatides by the cations, but no sufficiently critical data on this point exists. Great caution must be exercised in interpreting haemolysis data obtained in suspensions with differing salt solutions, as it is known that lysis can occur by ion-osmotic effects without the use of detergents or other lysins.[39] The second modifying factor of interest here is concerned with the role of the stroma cholinesterase. The

function of this enzyme as a possible controlling factor in the selective permeability of nerve cells has been studied for some time. The enzyme has also been suggested as of importance in the control of permeability in the erythrocyte. It has been shown by HOLLAND, GREIG and others[39], [40], [41] that acetyl choline retards haemolysis caused by bicarbonates and potassium salts, and that cholinesterase inhibitors acelerate haemolysis in these media, although complete correlation between permeability changes and the enzyme function is not yet proved.[42], [43] The possible function of cholinesterase in haemolysis by ionic detergents is not clear. There is little evidence that the enzyme plays anything but a subsidiary role in the process.[28], [29] ANDERSON[32] has shown that the enzyme is only slightly inhibited at haemolytic concentrations of several detergents. There is some evidence that acetyl choline retards, and prostigmine very slightly enhances, haemolysis by dodecylamine in normal saline and bicarbonate media, whereas there is a negligible effect for dodecyl sulphate lysis.[28], [32] As stated above, the alkyl homologues of acetyl choline fit the cholesterol penetration-haemolysis relation quite well.[28] The only evidence for supposing that the enzyme does play a minor part in detergent haemolysis is in the case of dodecylamine. Acetyl choline only effects the amine haemolysis in grossly unphysiological concentrations when the lysis is fast (high amine concentration). For slow amine lysis, the acetyl choline retardation is more marked, especially when the order of mixing is the addition of cell suspension to the mixture of acetyl choline and amine.[28], [29] It is possible that the effect in the dodecylamine case is due to the cationic group of the acetyl choline, and until further data on the effect of different cations on amine lysis is obtained, judgement on the interesting possible functions for cholinesterase in detergent haemolysis must be reserved.

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*The mechanism of haemolysis by non-ionic detergents*

The available data on non-ionic detergent lysins that do not penetrate cholesterol films indicate that they haemolyse by a non-specific detergency process.[2] The nature of the structures "deterged" from the stroma is unknown, and is unlikely to be identified by the techniques of surface chemistry. It is of interest to note in this connection that MITCHELL[45] has shown that butyl alcohol damages the cells of *Micrococcus pyogenes*, releasing acid-soluble phosphate, above a critical concentration which is almost identical with the critical haemolytic concentration of butyl alcohol.[2] The surface-chemical data on the haemolytic action of these non-ionic detergents do, however, provide some insight into the kinetics of the haemolytic reaction. Many of the kinetic equations used in the literature[9] to describe rates of haemolysis are of little value, as the physical significance of the various

"constants" used is obscure. It is only possible at present to describe the kinetics of such a complicated system in a broad semi-thermodynamic way. Each group of lysins may be expected to obey different rules. For example, bacterial lysins haemolyse at rates that vary linearly with concentration, pointing to an enzymic reaction at the stroma, whereas detergents haemolyse in a fashion indicative of a surface reaction of the type discussed above.[44] The non-ionic lysins will lyse completely at surface pressures above 34 dynes/cm.[2] It is an obvious inference that the particular value (34 dynes/cm) of the haemolytic surface pressure for the non-ionic lysins will depend on the choice of haemolytic end point.[2] The use of the 90-minute end point for complete lysis in conjunction with the theory that the non-ionic lysins "deterge" the stroma above a critical surface pressure of 34 dynes/cm means that the chosen lysis end point is attained on the prior condition of a favourable free energy gradient for "detergency" (i.e. surface pressure at least 34 dynes/cm). If other factors slow up the haemolysis at a given free energy gradient, the surface pressure at the 90 minute end point will be higher than 34 dynes/cm. Thus, given that the lysin is reacting, the surface pressure for the 90-minute end point should increase with molecular weight of the lysin, because of the decrease in the steric correction factor for the rate of reaction. Also, for two lysins of the same molecular weight (or more correctly, of the same steric factor) the lysin that is more surface active for a given concentration will lyse in 90 minutes at a higher surface pressure than the less surface active lysin, because of the lower collision rate factor for the first lysin. Both these predictions from elementary kinetic theory are borne out by the experiments of PETHICA and SCHULMAN[2] on alcohols and polyethers. As an example, it was found for the copolymer heptyl alcohol–11 ethylene oxide that a minimum in the surface tension curve corresponded with a maximum in the haemolytic activity (Fig. 3). With this compound, the effect of the variation of the collision factor on the haemolysis rate is thus shown up clearly. The substance haemolyses at the same rate with two different surface pressures, the lower concentration acting at a higher surface pressure, as predicted.[2] The interrelation of the monolayer penetration and simple detergency processes in haemolysis has been examined for sodium laurate. This substance fits equally well into both the model schemes under suitable circumstances.[2] By mixing taurocholate with laurate, the surface pressures of the laurate can be modified, and it has proved possible to separate the two possible modes of action of this lysin. In some circumstances the surface tension lowering is the more important property for haemolysis, and in others the cholesterol reaction predominates. It has also been shown that whereas ionic detergent lysins of opposite sign act by the cholesterol reaction process

when acting alone, they act by the surface pressure detergency process when mixed, and can be treated as non-ionic detergent lysins.[2] This arises from the formation of neutral cationic-anionic complexes between the detergents of opposite charge.

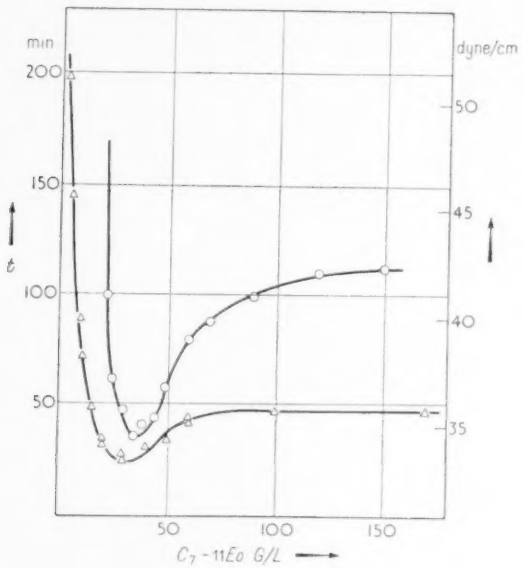


Fig. 3. The relation between haemolysis and surface tension lowering for heptyl alcohol—11 ethylene oxide at *p*H 7. △ Surface tension, ○ Haemolysis time. Data from [2].

*Antibiotic haemolysis*

There is little data available to judge the mode of action of haemolytic polypeptide antibiotics. The haemolytic activity of tyrocidin and gramicidin is reduced in the presence of alcohol-soluble stroma extracts,[46] and it appears likely that the action of these compounds on red cells is centred on lipid components of the stroma. Whether they show preference for phosphatides or other lipids is undetermined.

## The Surface Chemistry of Polypeptide Antibiotics and its Application to their Mode of Action

*Monomolecular films of polypeptide antibiotics*

During the past few years monolayers of synthetic polyamides and polypeptides have been studied both at the air/water and oil/water interfaces.[47], [48], [49] For the case of the naturally occurring antibiotic polypeptides very little published data is available. Certain of these



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substances can be spread as monolayers at the air/water interface in the presence of low concentrations of inorganic salts in the aqueous phase. However, the presence of high salt concentrations can stabilize partially soluble polypeptides at the interface, and can induce monolayer formation by polypeptides which are readily soluble in water. Fig. 4 shows the surface pressure or force/area curves for seven polypeptide antibiotics spread upon 70 per cent w/w ammonium sulphate solution and taken from the results of FEW and SCHULMAN.[50], [51]

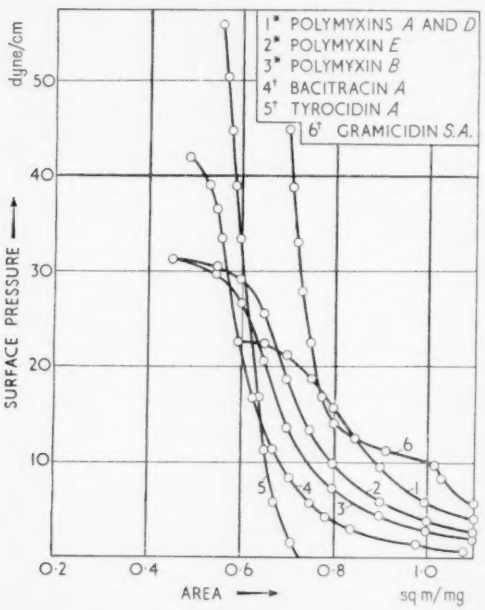


Fig. 4. Monomolecular films of polypeptide antibiotics spread upon 70 per cent w./w. $(NH_4)_2SO_4$ solution, at 20°. Data from [50] and [51]. Polypeptide samples from Dr. L. C. CRAIG† and The Wellcome Research Laboratories.*

These force/area curves indicate that the film forming molecules show a great variety of surface packing and intermolecular associations. Thus the monolayers of polymyxins A, B, D and E are all readily compressible and remain "liquid" films to the collapse pressure and therefore exhibit little, if any, intermolecular association. The monolayer formed by bacitracin A is again readily compressible, but the monolayer which is initially "liquid," "solidifies" at a surface pressure of 28 dynes/cm indicating strong intermolecular association as the film forming molecules are brought into close contact. Tyrocidin A forms a monolayer which is relatively incompressible and which solidifies at a surface pressure of only 1·5 dynes/cm. The monolayer obtained with gramicidin S.A. is initially liquid, but at a surface pressure of

10 dynes/cm the transition to a solid film commences and is accompanied by a rapid decrease of the monolayer area as it is subjected to further pressure. Solidification of the monolayer then takes place and the compressibility becomes similar to that of the tyrocidin A monolayer.

It has been suggested by ADAM[52] that the limiting area obtained with protein monolayers, when the polypeptide chains are closely packed at the interface, would be about 0·7 sq.m/mg. It can be seen from Fig. 4 that this limiting area is approximately achieved by the polypeptide antibiotics.

The pharmacological properties of the members of the polymyxin group of antibiotics have been investigated by BROWNLEE, BUSHBY and SHORT.[53] These authors have shown that polymyxins A, C and D are nephrotoxic when administered parenterally, whereas polymyxins B and E exhibit far less nephrotoxic action. A similar classification into two groups can be made with the monolayers of polymyxins A, B, D and E. Of these four polypeptides, polymyxins A and D have identical force/area curves characterized by a collapse pressure of 22·6 dyne/cm and a coefficient of minimum compressibility $\left(-\frac{1}{A}\frac{dA}{dF}\right)$ of 0·018 cm/dyne. Polymyxins B and E form significantly different monolayers when compared with those of polymyxins A and D. Whilst polymyxin B occupies a slightly smaller surface area than polymyxin E at all surface pressures, the curves are in other respects closely similar. Thus both the polypeptides have the same collapse pressure of 31·3 dyne/cm and the same coefficient of minimum compressibility of 0·013 cm/dyne.

The application of monolayer measurements for the determination of the molecular weights of the film forming molecules was introduced by GUASTALLA.[54], [55] The method depended upon the assumption that at very low surface pressures monolayers of certain substances show very little evidence of intermolecular attraction and behave in the manner of non-ideal two-dimensional gases. GUASTALLA was able to estimate the molecular weights of several proteins by the use of a two-dimensional gas law equation analogous to that employed for molecular weight determination from osmotic pressure data. The equation of state of an ideal two-dimensional gaseous monolayer can be written in the form $FA = RTW/M$,[56], [57] where $F$ is the surface pressure in dyne/cm, $A$ is the area in sq. cm, $W$ = weight of film spread in g, $M$ is the molecular weight, $R$ is the gas constant

$$= 8{\cdot}31 \times 10^7 \text{ Erg/Mol/Deg}$$

and $T$ is the absolute temperature. Assuming this equation applies

at zero surface pressure, when $A$ is expressed in $m^2$/mg the molecular weight can be determined from

$$M = \frac{RT}{\lim_{F=0} FA} = \frac{2435}{\lim_{F=0} FA} \text{ at } 20°$$

The value $\lim_{F=0} FA$ can be obtained by extrapolation of the $FA/F$ curves to $F = 0$.

This method has been investigated by BULL[58], [59] and ALLAN[60] using proteins and protein partial hydrolysates at the air/water interface, and by CHEESMAN,[61] using proteins at the liquid/liquid interface. Although in many cases the value of the molecular weight obtained from low surface pressure measurements on monolayers has agreed quite well with the values obtained in the bulk, in other cases the surface molecular weights have been only a small fraction of those determined by other methods. This appears to be especially so with measurements at the liquid/liquid interface. The apparently anomalous molecular weights at the oil/water interface have been discussed by DAVIES.[62] The method is not well suited for molecules of high molecular weight since as the molecular weight increases the surface pressures produced by the film molecules at a given surface density decreases, and the accuracy of their measurement decreases correspondingly. It would appear that the method finds best application for substances having a molecular weight not greater than 20,000. Low surface pressure measurements have been made on monolayers of polymyxins A, B, D and E by FEW and SCHULMAN.[50] Within experimental error, polymyxins A and D produced identical $F/A$ curves as in the case of the high pressure $F/A$ curves. Similarly, polymyxins B and E could be represented by a single $F/A$ curve over the pressure range studied. Extrapolation of the linear low pressure portion of the derived $FA/F$ curves to $F = 0$ gave intercepts indicating a molecular weight of $1220 \pm 60$ for polymyxins A and D, and $1280 \pm 70$ for polymyxins B and E. Thus there is no significant difference in the molecular weights of these four polymyxins; the mean molecular weight of 1250 is in good agreement with the value of 1150 obtained for the molecular weight of polymyxin D determined by the osmometric method.[63] The presence of a minimum in both derived $FA/F$ curves for polymyxin monolayers at a surface pressure of c.0·2 dyne/cm may indicate that intermolecular association takes place with these polypeptides. Extrapolation of the linear portions of both $FA/F$ curves to $F = 0$ using only surface pressure readings between 0·2 and 0·5 dyne/cm give limiting values of $FA$ suggesting molecular weights very close to twice the correct values. Similar minima were obtained by BULL[58] for zein monolayers at the air/water interface, and by

CHEESMAN[61] for bovine serum albumin monolayers at the benzene/water interface. It has, however, been pointed out by DAVIES that these minima can be explained in terms of charge effects without changes in the molecular weight.[62]

*The mode of action of polymyxin E, a cationic polypeptide antibiotic*

The effect of synthetic detergents upon bacterial metabolism and their bactericidal action have been investigated by BAKER, HARRISON and MILLER.[64], [65] In general, the anionic detergents were only bactericidal against Gram-positive organisms, whereas the cationic detergents were found to exhibit marked bactericidal effects towards Gram-positive organisms and somewhat less pronounced action on Gram-negative bacteria. BAKER, HARRISON and MILLER[66] suggested that, in all probability, the initial action of the detergents was to disorganize the cell membrane. This hypothesis was supported by HOTCHKISS,[67] who considered that the loss of intracellular components to the bacterial suspension medium subsequent to the damage of the cell would be sufficient to account for the depression or inhibition of bacterial metabolism. The cationic polypeptide antibiotic tyrocidin was also shown by HOTCHKISS[67] to exert a marked bactericidal action towards Gram-positive bacteria and to cause leakage of nitrogen and phosphorus-containing components from treated *staphylococci*. Subsequently GALE and TAYLOR[68] demonstrated that with tyrocidin treated *streptococci* amino-acids were lost from the internal environment of the cells, whilst the electron micrographs of tyrocidin-treated cells obtained by MITCHELL and CROWE indicated that the cell wall of the organisms had been ruptured.[69]

Members of the polymyxin group of cationic polypeptide antibiotics have been shown to be rapidly bactericidal and not merely bacteriostatic,[70], [71], [72] and electron micrographs of polymyxin-treated cells have been reported to be much less electron-dense than those of normal cells. Thus, whilst the polymyxin cationic antibiotics appear to exert a similar antibacterial action to that of the cationic detergents and tyrocidin, the polymyxins are in general only active towards the Gram-negative bacteria. It has been observed that the treatment of polymyxin solutions with washed resting suspensions of a susceptible bacteria resulted in a loss of antibacterial activity whereas similar treatment with a resistant organism caused no loss of activity.[72] This effect was interpreted as the result of absorption of the antibiotic upon the bacterial bodies of the susceptible organisms. It has been pointed out by HOTCHKISS,[67] for the case of synthetic surface active agents, that penetration of these substances into the internal environment of the cell would only be expected to be appreciable if accompanied by damage to the cell membrane. A similar state of affairs should hold



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in the case of the structurally complex polypeptide antibiotics, and thus a study of the absorption of these agents by susceptible and resistant bacteria should throw much light upon their mode of action.

The establishment of the conditions required for stable monolayer formation by the polymyxin antibiotics has provided a simple and accurate method for the assay of their solutions.[50] This technique has made possible the detailed investigation of the absorption of these agents by susceptible and resistant bacteria and bacterial components, and has been used to study the bacterial absorption of polymyxin E

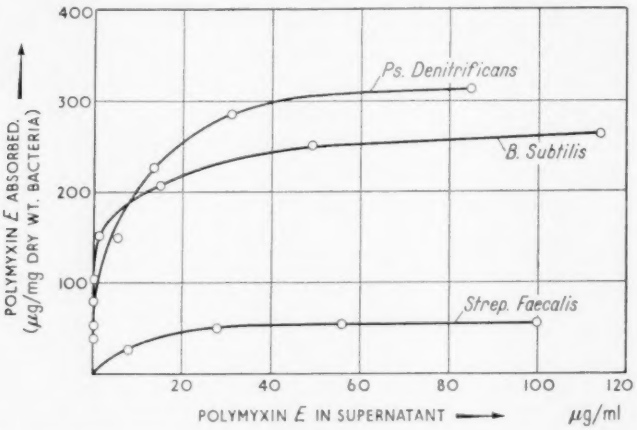


Fig. 5. Absorption of polymyxin E by bacteria. Organisms incubated at 25° for 20 minutes in 0·01 M phosphate buffer *p*H 6·3 containing polymyxin E. Data from [74].

by FEW and SCHULMAN.[73], [74] The release of cell solutes having an absorption maximum in their ultraviolet spectra at 260 m$\mu$ was followed concurrently with the absorption experiments using the method described by SALTON.[3] In all cases studied, the polymyxin sensitive bacteria were capable of absorbing rapidly 4–5 times as much polymyxin E as that bound by the resistant bacteria. In particular, at low concentrations of the antibiotic, bacterial absorption of the polymyxin E was complete with sensitive bacteria, whereas only poor absorption occurred in this region with resistant bacteria. Fig. 5 shows the absorption isotherms obtained after 20 minute contact at 25° with the antibiotic, for the sensitive organisms *Bacillus subtilis* and *Pseudomonas denitrificans* and the resistant organism *Streptococcus faecalis*. The addition of polymyxin E to suspensions of sensitive bacteria caused a rapid appearance of cell solutes in the supernatant fluids; the amounts released by the bacteria corresponded closely

with the quantities released by bacteria treated with bactericidal concentrations of cetyl trimethylammonium bromide observed by SALTON.[3] A similar release of cellular material, which had an absorption maximum at 260 m$\mu$, and of pentose and phosphate from a polymyxin-treated sensitive organism was observed by NEWTON.[75] Polymyxin-resistant bacteria, on the other hand, release initially only very small quantities of cell solutes having an absorption maximum at 260 m$\mu$.

The polymyxin E absorption isotherms given by the isolated bacterial cell walls, which were obtained by mechanical disruption of bacterial cells, were similar to those obtained for the intact organism.[74] The cell walls derived from sensitive bacteria are capable of absorbing much greater quantities of the antibiotic than those obtained from the resistant bacteria. Also, at polymyxin E saturation levels, a given dry weight of bacterial cell wall material absorbed similar quantities of the antibiotic as was absorbed by the same dry weight of intact organisms from which the cell walls were derived. Thus polymyxin E can absorb at sites both within the bacterial cell wall and internal environment of the cell.

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Although polymyxin sensitive bacteria are capable of absorbing large quantities of the antibiotic, complete saturation of the organisms was not necessary in order to kill them. More than 99·5 per cent mortality was obtained when the bacteria were only $\frac{1}{12}$th saturated with the antibiotic. At the low polymyxin concentrations required, the shapes of the absorption isotherms obtained for both intact bacteria and bacterial cell walls suggested that the distribution of the antibiotic would be such as to favour absorption at sites within the bacterial cell wall, with but little, if any, penetration into the internal environment of the cell. Since there is a rapid release of soluble cellular constituents from polymyxin treated sensitive bacteria it has been suggested that polymyxin E combines with, and thereby disorganizes, the structures responsible for osmotic pressure regulation within the bacterial cell wall. Evidence of extensive damage to the cell wall structures has been observed in the electron microscope.[76] The treatment of the isolated cell walls obtained from a sensitive bacteria with polymyxin E (Figs. 6 and 7) resulted in marked morphological changes, whereas similar treatment of the cell walls of a resistant bacteria caused no change in their appearance. Thus polymyxin E exerts an antibacterial action which is very similar to that of the synthetic surface active agents and, as was pointed out by HOTCHKISS,[67] is directly analogous to haemolysis by ionic detergents. The low and time dependent absorption of polymyxin E by resistant bacteria, which is due to its inability to pass readily through the cell wall and cellular membrane, would support this view.

*Polymyxin E—phosphatide associations*

Phosphatides such as lecithin, cephalin and sphingomyelin, have been shown to prevent the inhibition of bacterial metabolism and the bactericidal effects caused by anionic and cationic detergents. Such studies have been pursued to obtain information concerning the probable site of action of surface active agents and the role of phosphatides in cellular membranes. The protoplasmic membrane has been suggested to consist of a layer of lipid molecules between absorbed protein layers,[77] or a lipoprotein mosaic.[78] As pointed out by BAKER, HARRISON and MILLER,[66] a study of the antagonistic action of phosphatides derived from bacterial cells upon surface active agents, might be of value in elucidating a possible relationship between cellular phosphatides and the resistance or susceptibility of the bacteria.

Studies on the antagonistic effects of soybean lecithin and lipositol preparations upon polymyxin antibacterial activity have been made by BLISS, CHANDLER and SCHOENBECH.[72] Although slight antagonistic action was observed with the soybean preparations they appeared to be no more effective than similar concentrations of commercial soap. The effect of five lipid preparations upon polymyxin E activity has been investigated by FEW.[51] Samples of beef heart lecithin, cardiolipin and Ox red cell stroma cephalin (mainly phosphatidyl serine[79]) were studied together with two lipid preparations of bacterial origin. The bacterial lipids were obtained by treatment of the appropriate bacterial fraction first with 95 per cent methanol, to disrupt lipo-protein complexes,[80] followed by repeated extraction with diethyl ether. By this means, lipid amounting to 12·8 per cent by weight, and having a total nitrogen and phosphorus content of 1·1 per cent and 4·3 per cent respectively, was extracted from cell walls isolated from a polymyxin sensitive *Pseudomonas denitrificans*. A second bacterial fraction was obtained from a polymyxin resistant *Staphylococcus aureus*. The fraction was deposited by high speed centrifugation of the supernatant fluid obtained after disruption of the organisms and removal of the cell walls. It has been suggested that this small particle fraction so obtained forms a continuous layer beneath the cell wall of the intact *staphylococci* and may in fact constitute a lipid membrane.[81] From this small particle fraction 22·3 per cent lipid was extracted, the lipid having a total nitrogen and phosphorus content of 0·8 per cent and 1·8 per cent respectively.

The effect of these five lipids on polymyxin E bactericidal action was studied by adding the lipids to a final concentration of 25 $\mu$g/ml to solutions of polymyxin E. After an appropriate time interval suspensions of *Pseudomonas denitrificans* were added to the tubes and sub-cultures made after incubation of the bacterial suspensions for

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Fig. 6. Electron micrograph of mechanically disrupted cells of *Ps. denitrificans* × 32,000. Note the presence of a secondary uniform layer within the cell wall. Data from [76].

Fig. 7. Electron micrograph of mechanically disrupted cells of *Ps. denitrificans* after treatment with 500 $\mu$g/ml. of polymyxin E for 20 minutes at 25° × 32,000. Data from [76].

TABLE 1

*Effect of Lipids on Polymyxin E Activity*

Bacteria: *Ps. denitrificans* 0·01 mg dry wt/ml
0·01 M Phosphate buffer *p*H 6·3
Lipids at 25 μg/ml

| Polymyxin Conc. μg/ml | 0 | 0·31 | 0·63 | 1·25 | 2·5 | 5·0 | 10 | 20 | 40 |
|---|---|---|---|---|---|---|---|---|---|
| Control (no lipid) | + | + | + | + | − | − | − | − | − |
| Lecithin | + | + | + | + | − | − | − | − | − |
| Cardiolipin | + | + | + | + | + | + | + | −* | −* |
| *Ps. denitrificans* Cell wall lipid | + | + | + | + | + | + | −* | −* | −* |
| *Staph. aureus* small particle fraction lipid | + | + | + | + | + | − | −* | −* | −* |
| Cephalin | + | + | + | + | + | + | −* | −* | −* |

\+ denotes growth on subculture.
− no growth on subculture.
* precipitate formed in tubes before addition of bacteria.

20 minutes at 25°. The results obtained are shown in Table 1 and indicate that, with the exception of lecithin, all the lipids caused the bactericidal end point to be raised. In this respect the phosphatidic acid

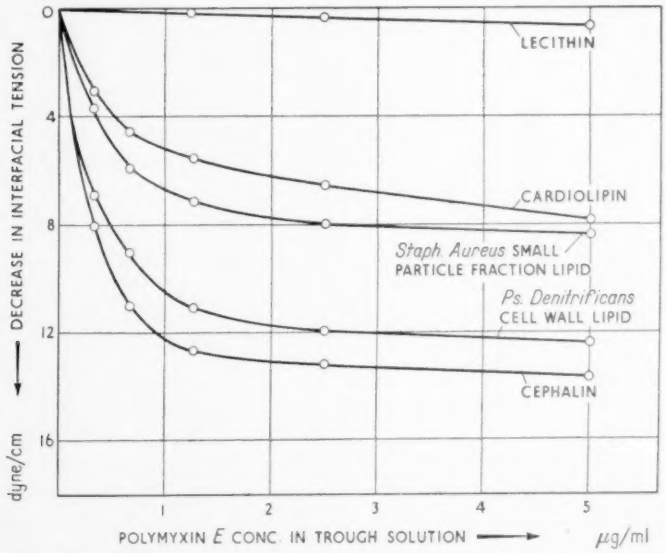


Fig. 8. Penetration of lipid monolayers by polymyxin E. Lipids spread upon 0·01 M phosphate buffer *p*H 6·8, compressed to 5·6 dynes/cm and polymyxin E added to the trough solution [51].

cardiolipin was most effective. At the higher concentrations of polymyxin E, definite insoluble complex formation was observed with all

lipids with the exception of lecithin. Similar insoluble complex formation between unspecified phosphatides and polymyxin has been reported by LATTERADE and MACHEBOEUF.[82]

The reaction of spread monolayers of the lipids with polymyxin E injected into the solution underlying the lipids has also been studied using the constant area penetration technique.[83] The penetration isotherms obtained (Fig. 8) show that, with the exception of lecithin monolayers, the lipid monolayers are readily penetrated by polymyxin E, the greatest decrease in interfacial tension occurring with monolayers of cephalin and lipid from the cell walls of *Pseudomonas denitrificans*.[51]

Thus definite evidence of complex formation between polymyxin E and certain lipids has been obtained both when the lipids are orientated at a phase boundary and in aqueous dispersions. In particular it has been demonstrated that the lipid derived from the cell walls of the polymyxin sensitive *Pseudomonas denitrificans* has a greater reactivity with polymyxin E than the lipid obtained from the bacterial fraction isolated from the polymyxin resistant *Staphylococcus aureus*. However, there is as yet no direct evidence that the primary site of attack by polymyxin is located at the lipid structures associated with the bacterial cell wall.

### THE MODE OF ACTION OF SURFACE ACTIVE AGENTS IN RELATION TO THE STRUCTURE OF THE BACTERIAL CELL

Our knowledge of the nature of the surface of the bacterial cell has been derived from studies of electrophoretic mobilities of bacteria, the isolation and chemical analysis of immunologically active components, staining reactions, the action of lytic agents and studies of the osmotic behaviour of bacteria. The phenomenon of lysis of bacterial cells (bacteriolysis) is the clearest example of a change having been brought about in the cell surface. Thus the enzyme, lysozyme, causes a rapid dissolution and complete clearing of suspensions of sensitive bacteria. The lytic action of lysozyme has been shown to be the result of digestion of the bacterial cell wall[84], [85], [86], [87], [88] and the action of this enzyme is directed towards those organisms possessing the specific substrate in the cell wall.[89], [90] The nature and extent of lysis does, however, vary widely from one lytic substance to another and from one bacterium to another. Surface active compounds such as the quaternary ammonium salts differ markedly from lysozyme in their action on bacteria; they initiate a leakage of substances from the cells without effecting a complete dissolution or clearing of suspensions of susceptible bacteria. HOTCHKISS drew an analogy between haemolysis of red blood cells by surface active agents and the cytolytic effect of such agents on bacteria.[67], [91] This analogy was based on the release of nitrogeneous and phosphorus-containing substances into

the suspending fluid when bacteria were treated with certain surface active compounds in bactericidal concentrations.

The marked germicidal activity of the quaternary ammonium surface active agent "Zephiran" reported by DOMAGK[92] stimulated wide interest in the bactericidal properties of these compounds. Both cationic and anionic surface active substances have since been studied and of the two classes of compounds the former possesses stronger germicidal powers than the latter. Non-ionic detergents do not possess bactericidal activity.[92] Gram-positive bacteria are killed by both the cationic and anionic compounds, whereas Gram-negative organisms are killed only by the cationic surface agents.[65], [93], [94] In general the Gram-positive organisms are more susceptible to the cationic compounds than are the Gram-negative bacteria. Where homologous series of quaternary ammonium compounds have been studied the maximum bactericidal efficiency has been observed with the $C_{16}$ compounds.[95]

Various explanations have been proposed to account for the bactericidal action of surface active agents. KUHN and BIELIG[96] observed that germicidal concentrations of some compounds corresponded to concentrations effecting denaturation of proteins. BAKER, HARRISON and MILLER[66] found that bacteria could be protected from the bactericidal action of cationic surface active agents by prior exposure to phospholipids. These authors therefore suggested that a reasonable hypothesis to explain the rapid action of synthetic surface active compounds on bacteria would be based upon a disorganization of the cell membrane and denaturation of certain proteins essential to metabolism and growth. HOTCHKISS provided more direct evidence supporting the hypothesis of cell membrane disorganization when he demonstrated the leakage of nitrogenous and phosphorus-containing substances from bacteria treated with various surface active compounds.[67], [91] GALE and TAYLOR[68] showed that the free glutamic acid found inside the cells of *Streptococcus faecalis* was rapidly released when the organism was treated with bactericidal amounts of the basic polypeptide, tyrocidin. A similar cytolytic effect was also observed with other cationic and anionic surface active compounds and also with phenol.[68] By growing *Staphylococcus aureus* and *Pseudomonas fluorescens* on a medium containing radio-active phosphorus ($P^{32}$) SALTON[97] showed that there was an increased leakage of $P^{32}$ into the suspending fluid when "Cetavlon" (cetyltrimethyl ammonium bromide) was present. All of these observations provided abundant experimental evidence in support of the suggestion that surface active agents act on some structure or structures responsible for the retention of certain nitrogenous and phosphorus-containing substances within bacterial cells.



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*The nature of some cellular constituents and the course of their release from bacteria treated with cetyltrimethyl ammonium bromide*

GALE and TAYLOR[68] showed that the release of free glutamic acid from *Streptococcus faecalis* treated with tyrocidin occurred quite rapidly. However, as Gram-negative bacteria do not generally contain free amino-acids within their cells,[98] some other method had to be found in order to carry out comparative studies of the rate of release of cellular constituents from bacteria treated with surface active compounds. As bacteria have long been known to contain free purines, pyrimidines, nucleotides, etc. it seemed likely that they might be released from bacteria killed with surface active compounds and that they should be detectable by their ultraviolet absorption. For such studies cetyltrimethyl ammonium bromide (CTAB) proved ideal because of its exeeedingly low absorption in the ultraviolet wavelengths and because of its activity against both Gram-positive and Gram-negative bacteria. An examination of the ultraviolet absorption spectra of supernatant fluids from bacteria suspended in distilled water and bacteria suspended in solutions of CTAB showed absorption maxima at a wavelength of 260 m$\mu$[3]. Bacteria suspended in distilled water alone, showed a steady leakage of 260 m$\mu$-absorbing material into the suspending fluid. The presence of bactericidal concentrations of CTAB brought about a rapid release of the 260 m$\mu$-absorbing material and the level of material released was considerably greater than that released from untreated cells.[3] Both Gram-positive and Gram-negative bacteria showed a release of 260 m$\mu$-absorbing material, thus providing a sensitive method for following the leakage of substances from the cells into the suspending medium. By means of filter-paper chromotography the 260 m$\mu$-absorbing material released from CTAB-treated *Staphylococcus aureus* was shown to consist of a mixture of guanine, adenine, adenine riboside and uracil.[3]

That the course of release of 260 m$\mu$-absorbing material from CTAB-treated bacteria was a reflection of the release of other cell solutes was readily shown from experiments in which the release was followed concomitantly with the release of glutamic acid and inorganic phosphorus. For the two Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus faecalis* a parallel course of release of all three constituents (260 m$\mu$-absorbing material, inorganic P and glutamic acid) was established.[3] The course of release of the three cellular constituents from *Streptococcus faecalis* treated with a bactericidal amount of CTAB is illustrated in Fig. 9. The Gram-negative organism *Escherichia coli* showed a parallel release of inorganic P and 260 m$\mu$-absorbing material on treatment with CTAB[3]. 260 m$\mu$-absorbing material was also released from another Gram-negative organism

*Salmonella pullorum*.[3], [99] Thus when Gram-positive and Gram-negative bacteria were treated with sufficient CTAB to kill 99 per cent of the cells within 5–10 minutes, a rapid release of amino-acids, inorganic P, purine and pyrimidine compounds occurred. The amounts of such constituents appearing in the supernatant fluids generally reached maximum values within 2–3 hours at room temperature. Furthermore, the amounts of certain constituents released by killing bacteria with CTAB were comparable to the amounts released from heated bacteria.[3]

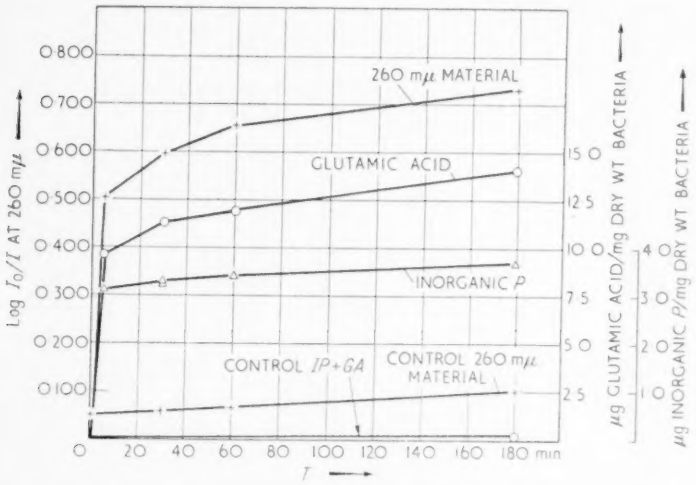


Fig. 9. Release of cellular constituents from *Strep. faecalis* (1·48 mg dry weight bacteria/ml) treated with 45 μg CTAB/ml at 20°C.[3] + 260 mμ absorbing material. ○ glutamic acid. △ inorganic phosphorus.

It is of some significance that the supernatant fluids from bacteria treated with enough CTAB to initiate the rapid leakage as shown in Fig. 9 give negative Biuret tests. This evidence supports the suggestion from the above observations that mainly low molecular weight cell solutes are released and that larger molecules such as proteins and nucleic acids are not released during the initial leakage process induced by CTAB. In this respect the cytolysis of bacteria treated with surface active agents such as CTAB would appear to differ from haemolysis However, it is clear that the initial rapid leakage reaching a maximum in 2–3 hours may be followed by a slower release of cellular components from *Staphylococcus aureus* treated with CTAB and held at 37°.[3] The latter process obviously involves autolytic breakdown of cell components as demonstrated by a progressive release of pentose and 260 mμ-absorbing compounds, a loss in cell weight and Gram-stain reaction.[3] A somewhat similar effect was observed when NEWTON[75] treated a polymyxin-sensitive strain of *Pseudomonas aeruginosa* with CTAB.



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As this organism showed a considerable degree of rupture and release of large amounts of 260 m$\mu$-absorbing material when suspended in distilled water, the release of 260-m$\mu$-absorbing constituents induced by CTAB was performed in a 1 per cent sodium chloride suspending medium. Treatment of this strain of *Pseudomonas aeruginosa* with CTAB under these conditions gave a release of 260 m$\mu$-absorbing material in 30 minutes; continued incubation of the treated suspensions for periods of up to 8 hours showed release of considerably more 260 m$\mu$-absorbing constituents, presumably due to autolytic systems involving the breakdown of nucleic acids.[75]

*Quantitative relationship between proportion of cells killed, amount of cationic compound and release of cell solutes*

A quantitative relationship between the proportion of cells killed by different amounts of tyrocidin and the amount of glutamic acid released from *Streptococcus faecalis* was observed by GALE and TAYLOR.[68] It was therefore not surprising that a similar quantitative relationship was observed when both Gram-positive and Gram-negative bacteria were treated with bactericidal amounts of CTAB below that required for 99 per cent killing. The leakage of 260 m$\mu$-absorbing material in 5 minutes was proportional to the percentage of cells killed and the percentage of bacteria killed was proportional to the amount of CTAB present.[3] This further illustrates the close time relationship between cell death and the initiation of leakage of low molecular weight substances from the cells.

*Electron-microscopy of cells treated with surface active compounds*

That surface active compounds bring about a rapid disorganization of the treated cells has been demonstrated by cytological studies. Evidence of cytolytic damage to tyrothricin-treated cells of *Bacillus cereus* was first shown by JOHNSON.[100] DYAR,[101] MITCHELL and CROWE[69] and MEISEL and UMANSKAYA[102] have provided further evidence of cellular disorganization and cytoplasmic damage resulting from treatment of bacteria and yeasts with surface active cationic agents. Some of these earlier observations were not sufficiently detailed to give critical evidence which might show whether treatment of bacteria with just sufficient cationic compound to give maximum killing involved cell wall damage in addition to cytoplasmic contraction. Treatment of bacteria with an amount of CTAB effecting 99 per cent mortality of the cells resulted in a marked contraction of the cytoplasm from the outer cell wall.[103] The cell wall itself showed no evidence of rupture although under these conditions cell solutes had been released. When *Escherichia coli* was treated with amounts of CTAB below that required for 99 per cent killing there was a correlation between the number of cells killed and the

proportion of cells in which the cytoplasm had contracted from the outer wall. Thus cell death, contraction of the cytoplam and release of cell solutes occur concomitantly. From such studies it seems reasonable to conclude that damage to the permeability barrier as shown by the leakage of cell solutes does not involve any detectable change in the appearance of the outer cell wall structure as seen in the electron microscope. It is of course conceivable that cell wall damage may occur and that the changes are beyond the limits of resolution of the electron microscope.

When cells were treated with amounts of CTAB greatly exceeding that required for maximum killing, damage to the cell wall structures was apparent.[103] However, as the isolated cell walls of some of the organisms used in these studies are insoluble in high concentrations of CTAB[99] it would appear that the damage to the cell wall structures initiated by treatment of cells with large amounts of CTAB involves autolytic systems present in the organisms. As the ratio of weight of cationic compound to weight of cells treated is of prime importance in determining the sequence of events such as death, leakage of cell solutes[3], [68] and contraction of the cytoplasm[103] it becomes more difficult to interpret the cytological changes where these factors have not been controlled. For example, the extensive cytological damage to two Bacillus species recently shown by CHAPLIN[104] would suggest that the amount of quaternary ammonium compound greatly exceeded the minimum amount required for maximum killing.

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*Adsorption of surface active compounds by bacteria*

It has been known for some time that bacteria possess a marked affinity for certain surface active substances. This is especially so for positively charged quaternary ammonium compounds.[105] Electrophoretic studies have shown a neutralization and reversal of the negative charge of bacterial cells treated with the cationic compounds.[106], [107], [108] These studies have suggested that a cationic substance such as CTAB is rapidly adsorbed by bacteria. This indeed was not surprising in view of the rapidity with which these agents kill susceptible bacteria. Quantitative estimations of the uptake of cationic surface active compounds by bacteria clearly show that the cells possess a capacity to adsorb amounts of these substances well above the amounts which exert maximum killing. MCQUILLEN found that the uptake of CTAB by *Staphylococcus aureus* at saturation amounts to about $\frac{1}{3}$ of its own dry-weight.[108] Salton reported that saturation of *Staphylococcus aureus* and *Pseudomonas fluorescens* with "Cetavlon" (CTAB) at *p*H 8·2 corresponded to an uptake of $1{\cdot}3 \times 10^8$ and $2{\cdot}2 \times 10^8$ molecules per cell respectively.[97] The amounts of CTAB adsorbed at saturation levels varied from one organism to another but there was no

relationship between susceptibility and the amount of CTAB adsorbed.[3] Thus the maximum amount of CTAB adsorbed by *Streptococcus faecalis* was approximately the same as that adsorbed by the more resistant organism, *Escherichia coli*.[3]

It is of some interest to consider the adsorption of surface active agents in terms of cell surface area. ALEXANDER and MCMULLEN[107] found that the amount of the anionic surface-active compound Aerosol OT adsorbed by bacteria corresponded to about a monolayer, whereas the adsorption of cationic compounds greatly exceeded the amount corresponding to a monomolecular film. If the amount of CTAB taken up at saturation by *Staphylococcus aureus* was spread as a closely packed monolayer it would occupy an area of approximately 15 times the cell surface area (assuming a sphere of 1 $\mu$ in diameter) whilst the corresponding ratio for *B. pumilis* was 18 times the cell surface area.[3] VALKO and DIBBLEE[109] studied the adsorption of dimethyl-dodecyl benzylammonium chloride by *Escherichia coli* and they concluded from the adsorption data that *Escherichia coli* had about the same equivalent weight as many animal proteins. These investigations[3], [105], [107], [108], [109] therefore suggest that both surface and internal components of the bacterial cell are capable of combining with or binding cationic surface active compounds.

*The nature of bacterial cell walls*

Until recently our knowledge of the chemical constitution of the rigid cell-wall structures of bacteria has been fragmentary, and the extent to which the bactericidal action of surface active compounds involved an interaction with cell wall components could not be assessed. With the development of suitable procedures for the isolation of cell walls[110], [111] it has been possible to obtain direct information about the chemical nature of these structures and to study the action of certain surface active compounds on the isolated walls.

By mechanical disintegration of bacteria in the Mickle disintegrator SALTON and HORNE[110] showed that subsequent differential centrifugation gave cell wall fractions relatively free of electron-dense cytoplasmic materials. Cell walls of several Gram-negative bacteria were also prepared by heat-treatment rupture followed by differential centrifugation, but the yields of cell wall material were rather low. To obtain sufficient quantities of isolated cell walls for chemical analysis the procedure involving mechanical disintegration of bacteria with minute glass beads in the Mickle disintegrator was used, and the composition of the cell walls of a number of Gram-positive and Gram-negative bacteria has now been ascertained.[99], [112], [113], [114] Several other investigators have studied the composition of bacterial cell walls. WEIDEL[115] has examined the cell wall of *Escherichia coli* prepared by

autolysis and enzymic digestion of the organisms, MITCHELL and MOYLE[81] investigated the "envelope" of *Staphylococcus aureus* and HOLDSWORTH[116] the cell wall of *Corynebacterium diphtheriae*.

It is now evident from these recent investigations that the composition of the cell wall varies considerably from one organism to another. The cell wall of *Escherichia coli* investigated by WEIDEL[115] was essentially of lipo-protein nature, whilst MITCHELL and MOYLE[81] found the "envelope" of *Staphylococcus aureus* to be a phosphoglycero-protein complex. On the other hand the cell wall of *Streptococcus faecalis* was composed mainly of carbohydrate (60 per cent) and an amino-acid-containing moiety.[99], [112] The cell wall of *Corynebacterium diphtheriae* studied by HOLDSWORTH[116] possessed both carbohydrate and protein components. A more extensive range of organisms has now been studied by SALTON,[113], [114] and several outstanding differences between the composition of the cell walls of Gram-positive and Gram-negative bacteria has now become apparent.

The cell walls of two Gram-negative bacteria contain considerably more lipid than do the walls of the Gram-positive bacteria. In addition to the marked differences in the lipid contents of the cell walls from the two groups, the amino-acid constitution of the walls from Gram-positive bacteria differs from that of the walls of the Gram-negative organisms.[113], [114] The cell walls of the Gram-positive bacteria are characterized by a limited amino-acid constitution, the absence of

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TABLE 2

*Properties of the Cell Walls of Some Gram-positive and Gram-negative Bacteria*[112], [114]

| *ORGANISM* | *Characteristics of the Amino-acid Constitution* | *Number of known amino-acids identified by chromatography* | *Percentage dry wt cell wall* | |
|---|---|---|---|---|
| | | | *Hexosamine content* | *Lipid content* |
| GRAM-POSITIVE | | | | |
| *Strep. faecalis* | Absence of aromatic, certain S-containing amino-acids, arginine and proline | 9 | 22·2 | 2·3 |
| *Strep. pyogenes* | | 9 | 11·8 | — |
| *M. lysodeikticus* | | 4 | 16·1 | 1·2 |
| *Sarcina lutea* | | 5 | 16·3 | 1·1 |
| *B. subtilis* | | 10 | 8·5 | 2·6 |
| GRAM-NEGATIVE | | | | |
| *Escherichia coli* | Presence of aromatic, S-containing amino-acids, arginine and proline | 17 | 3·0 | 20·8<br>22·6 |
| *Salmonella pullorum* | | 16 | 4·8 | 19·0 |

aromatic and sulphur-containing amino-acids being particularly conspicuous. A much more complete range of amino-acids, including aromatic, certain sulphur-containing amino-acids, arginine and proline was found in the cell walls of several Gram-negative bacteria. Both Gram-positive and Gram-negative bacteria contained hexosamine in their cell walls but the quantities were rather higher in those of the Gram-positive bacteria. Some of the differences in the composition of cell walls from organisms of both groups are summarized in Table 2 shown on previous page.

*The action of surface active agents on isolated cell walls of bacteria*

Although our knowledge of the composition of the bacterial cell wall is still rather scant and the range of organisms so far used in these investigations is very limited, some tentative suggestions as to the manner in which the cell walls may influence the action of surface active agents seem appropriate. The most obvious relationship between the composition of cell walls and the susceptibility of bacteria to CTAB is that the two Gram-negative bacteria possessing walls with high lipid contents are more resistant than the two Gram-positive bacteria with walls of low lipid content. This relationship is illustrated in Table 3

TABLE 3

*Comparison of the Susceptibility of Bacteria to CTAB with the Lipid Content of the Isolated Cell Walls*

| *ORGANISM* | *Weight of cells killed by 45 μg CTAB in 30 minutes* (data from[3]) | *Lipid content of cell walls as percentage dry wt cell wall* |
|---|---|---|
| *Strep. faecalis* . . | 1·45 mg. | 2·3 |
| *Staph. aureus* . . | 1·12 | 2·8 |
| *Esch. coli* . . | 0·8 | 22·6 |
| *Salmonella pullorum* . | 0·93 | 19·0 |

by the data taken from SALTON[3] (the order of susceptibility is shown by calculating the weight of cells killed by 45 μg CTAB in 30 minutes) together with the determinations of the lipid contents of the walls of these organisms.

It is conceivable that one of the reasons why Gram-negative bacteria are generally less susceptible to the bactericidal action of the cationic surface active compounds is that the high lipid content of their walls offers a more effective barrier to the penetration of compounds such as CTAB. The complete resistance of Gram-negative bacteria to the rather weaker anionic surface-active bactericidal agents may also be related to the nature and quantity of lipid in the cell walls of these organisms.

Some recent work by CHAPLIN[118] supports the view that lipid substances of the cell surface are associated with resistance to quaternary ammonium compounds. CHAPLIN concluded that the acquired resistance of *Serratia marcescens* to alkyldimethylbenzyl ammonium chloride was dependent upon the increased lipid content of the resistant cell.

With the methods now available for the preparation of cell walls some of these ideas may now be tested experimentally. In some unpublished observations by SALTON[117] the adsorption of CTAB by

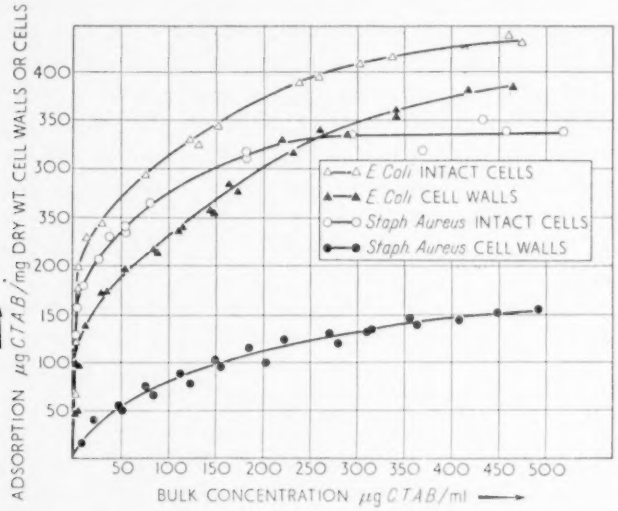


Fig. 10. Adsorption of CTAB by intact cells and isolated cell walls.[117], [3] *Esch. coli* △ intact cells, ▲ cell walls; *Staph. aureus* ○ intact cells, ● cell walls.

cell walls from *Staphylococcus aureus*, *Streptococcus faecalis* and *Escherichia coli* was studied. The adsorption curves for the uptake of CTAB by intact cells of *Staphylococcus aureus* and *Escherichia coli* (data from SALTON[3]) was compared with that for isolated cell walls of the two organisms and these are so shown in Fig. 10. Although there was a greater difference in the levels of saturation of intact cells and cell walls with *Staphylococcus aureus* than with *Escherichia coli*, the difference in the saturation levels of intact cells and cell walls of the more susceptible *Streptococcus faecalis* was of the same order as that found for *Escherichia coli*. It may well be that the amounts of CTAB firmly held by the cell walls of Gram-positive and Gram-negative bacteria would be more revealing than the amount required to saturate the cell walls. Unpublished observations[117] in which *Staphylococcus aureus* cell walls were saturated with either CTAB or cetylpyridinium chloride (CPC) and subsequently washed have shown that virtually



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all of the adsorbed CTAB or CPC may be removed. This finding is very similar to the results obtained in adsorption studies of alkyl dimethylbenzylammonium chloride on spores of *Bacillus subtilis*.[119] These authors were able to wash the spores free of alkyldimethylbenzylammonium chloride, thereby restoring the normal charge of the spores and removing the inhibitory effect of the agent on spore germination. Comparable experiments have yet to be performed on the cell walls of Gram-negative bacteria. Such experiments should give a good indication of the extent to which the cell wall lipid of these organisms confers a greater resistance to the bactericidal action of the surface active cationic compounds. At least it is now clear that the cell walls of *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus faecalis* are not dissolved by treatment with high concentrations of CTAB. Some authors[120] have suggested that the inhibition of a detergent-sensitive enzyme would explain the action of these cationic agents on bacteria but most of the experimental evidence so far accumulated is in complete agreement with the hypothesis that these agents effect an irreversible change in the properties of the permeability barrier of bacterial cells.

A particularly promising means of investigating the action of surface active compounds on the cell membrane has now become apparent. The fact that lysozyme brings about a specific depolymerization of the cell walls of certain sensitive organisms led WEIBULL[121] to investigate the digestion of the cell walls of intact *Bacillus megaterium* under controlled conditions in a sucrose medium. Dissolution of the cell walls of the organism under these conditions gave spherical protoplasts. WEIBULL showed that the protoplasts, deprived of cell walls, were capable of metabolizing glucose at the same rate as intact cells.[121] He further showed that there was no significant leakage of 260 m$\mu$-absorbing material from the protoplasts, thus suggesting that the protoplasts membrane is intact and is responsible for the retention of low molecular weight substances in the cells. Some preliminary experiments on the action of CTAB on the protoplasts of *Bacillus megaterium* have been undertaken[117] and the results indicate that this approach to the problem of the action of surface-active agents will provide a clearer understanding of the mode of action of these compounds on the bacterial cell.

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# THE DIRECT ACTION OF IONIZING RADIATION ON ENZYMES AND ANTIGENS*

*E. C. Pollard, W. R. Guild, F. Hutchinson and R. B. Setlow*

## Introduction

The discovery that enzymes can be inactivated by ionizing radiation is by now quite old. Pioneer work on enzyme preparations in solution was conducted by Hussey and Thompson[1] and Northrop.[2] In these experiments enzymes in solution were bombarded by particles from radon or by x-rays and the loss of activity of the enzyme measured. The survival ratio was found, as for other biological inactivations, to be a logarithmic function of the total radiant energy supplied.

Attempts to use such a relation to determine a consistent inactivation volume, or volume within which energy must statistically be delivered by radiation for inactivation, failed. The reason became apparent after the work of Fricke,[3] who showed that water could become activated by radiation and that the chemical action resulting from this could play a very important part in the enzyme destruction. In view of this discovery, use of the physical distribution of energy resulting from radiation to measure sensitive volumes was not attempted, with one exception. Svedberg and Brohult[4] irradiated hemocyanin with alpha rays, found a splitting of the molecule as seen in the ultracentrifuge, and used the quantitative dose relationship to estimate the molecular weight for the protein molecule. The estimate was reasonable and succeeded for two reasons. The first was the fact that alpha rays, with dense track ionization, produce relatively little activation of water, so that the indirect action discovered by Fricke was at a minimum. The second was the observation of actual molecular disruption, which is more likely to be due to dense localized action than to the chemical effects of activated water.

The apparent success of ionizing radiation in estimating virus dimensions[5], [6] as determined by measuring the inactivation volume for randomly distributed energy deposition and the plausible application to gene size measurements,[7] led Lea to consider the desirability of checking radiation determined sensitive volumes against known molecular sizes. In a pioneer paper, Lea, Smith, Holmes and

* Much of the experimental work here described was supported by the U.S. Atomic Energy Commission.

MARKHAM[8] observed the effect of intense X-ray bombardment on myosin and ribonuclease and from the loss of activity determined the inactivation volume. The agreement with the known molecular volumes was fair and indicated promise to the method. In order to eliminate the indirect effect of activated water, these authors performed the irradiation in the dry state.

LEA's reasoning was as follows.[9] The observed inactivation follows the relation

$$n/n_0 = e^{-CD}$$

where $n/n_0$ is the ratio of activity remaining after a dose $D$. The quantity $C$ depends on the enzyme. For myosin it is much larger than for ribonuclease and this difference, considered quantitatively, is the basis for estimating molecular sizes. In making a quantitative estimate, the first decision which is needed concerns the molecular sensitivity. LEA advanced the hypothesis that one ionization produced anywhere in an enzyme molecule would remove its biological function. With this assumption as a start one next has to know the spatial distribution of ionizations. From cloud chamber data, LEA concluded that ionizations were located close to the *primary ionization* in clusters of three ion pairs on the average. So that while one ionization suffices to inactivate a molecule, in many cases two, three, or even more, will be available. It will be seen later that refinement of this idea is quite possible and indeed necessary.

From the measured dose in roentgens, the known charge on an ion, and the relative density of air and protein, LEA deduced the number of clusters per unit volume of the enzyme sample. Call this number $I$ for any one dose. Now simple probability considerations show that if the sensitive volume is $V$, the probability of one cluster falling in $V$ is $VI$ and the probability of no cluster is given by the Poisson formula as $e^{-VI}$. We thus have the proportion of enzyme undamaged, which is an experimental measure of this probability, given by

$$\frac{n}{n_0} = e^{-VI} \qquad \ldots.(1)$$

Relating this to the experimental data, we can readily find $V$. If LEA's hypothesis is tenable, then $V$ should be related simply to the molecular size.

To test further the validity of this remarkably simple idea that one ionization inactivates one molecule a series of irradiations of enzymes, antigens and toxins has been made in the Yale Biophysics Laboratory. Some of the results of this study have been described by POLLARD.[10] The broad conclusions of the work favour the hypothesis of LEA, but as will be seen, the hypothesis is in need of elaboration if quantitative

results are to be deduced. Also, there is clear evidence for the effect of factors other than ionization on radiation sensitivity.

In what follows more recent work on enzyme inactivation will be described and the new factors emerging, namely critical energy sensitivity, thermal influence on sensitivity and the effect of physical attachment to other substances, will be discussed.

Since it is unsatisfactory to present a wholly empirical approach, an attempt will be made to provide a physical explanation for the results found. This approach, as will be seen, is still incomplete.

## Experimental Considerations

The interpretation of the results of the irradiation of biological molecules in terms of a physical process depends crucially on a knowledge of the spatial distribution of the sites of energy release.[9], [10] The simplest case is that in which the material is dry, with no opportunity for activated water molecules to diffuse through the sample and spread the effects of the ionizing radiation. Working with dry preparations of most biological molecules is not hard, and nearly all the work covered in this review has been done in this state.

The types of radiations most used in biological irradiations—x-rays, gamma rays, beta rays, and neutrons—are characterized by a wide spread in the energy distribution of the charged particles (electrons and recoil nuclei) which are the ultimate creators of the ionization produced. This makes the calculation of the spatial distribution of the ionization a difficult task. To eliminate this difficulty most of the irradiations reported here have been done with fast charged particles—deuterons, alpha particles, and electrons.

Heavy charged particles such as deuterons or alpha particles create ionization in dense columns along the straight tracks of the particles. Irradiation with such rays may be likened to probing the structure with red-hot needles. On the other hand, electrons with energies of several Mev have their primary ionizations separated by 4000–5000 Å. For molecules which have dimensions less than this, the primary ionizations are produced at random throughout the volume, more randomly than by almost any other type of radiation. Use of these two entirely different types of radiation gives complementary information on molecules, as will be discussed later.

Another reason for using charged particles for the irradiation of biological molecules is the very large doses required. To put an average of one primary ionization into a spherical molecule of molecular weight 100,000 requires the order of $10^7$ roentgens. Such a dose may be given by a 2 Mev electron beam of intensity 1 microampere/sq.cm in 60 seconds. The equivalent inactivating dose for the same molecule with 4 Mev deuterons is 0·01 microampere/sq.cm for 80 seconds.

Beryllium window x-ray tubes which will deliver very soft radiation at rates up to a million roentgens/min. are available, but do not seem to have been used much for this type of work.

## Experimental Technique

The experimental arrangement for deuteron and alpha particle irradiations is shown in Fig. 1. The bombardment chamber is at the end of a brass pipe about four feet long which is connected to the exit port of an accelerator. The beam enters this pipe through a thin aluminium

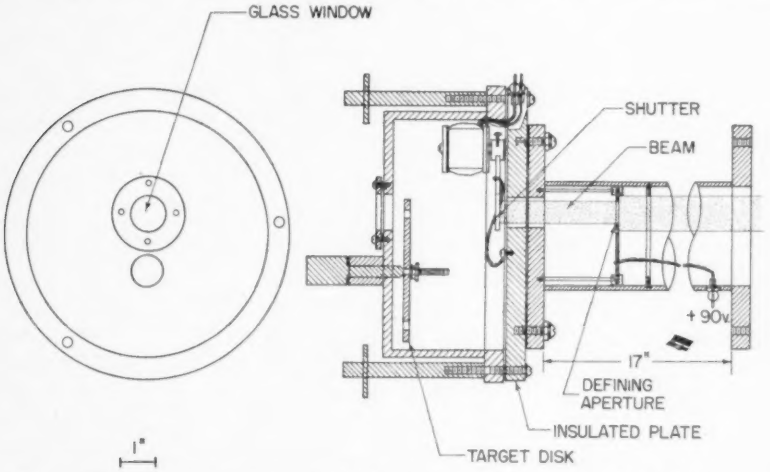


Fig. 1. Experimental arrangement for deuteron and alpha particle irradiation.

foil, which serves as a diffuser by scattering the beam. The deuterons are next incident on a copper disc, with a circular aperture. The disc is insulated and kept at 90 volts positive. The part of the beam which goes through the circular aperture then impinges either on a brass shutter, or on the sample holder, depending on whether the shutter is open or not. The shutter is operated electrically by batteries on an insulating stand. The whole end is insulated, partly by a rubber gasket and partly by insulating sleeves surrounding the bolts which fasten the end plate in place. This whole end then serves as a Faraday cage to collect the beam. The beam is normally measured with a galvanometer as shown. To check the current readings, the beam was also measured on one occasion by thermal methods, using the amount of liquid air evaporated as an indicator. The check was good.

The procedure in a run is as follows. The sample is brought into place and the beam turned on from the control room. When it has settled down to a steady value the shutter is opened and a stopwatch started. The dose is then measured as average current times time, or



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alternatively an electronic integrator is used. Both systems are satisfactory. The next sample is then brought in, and so on. Some radioactivity develops in the samples in a deuteron run. If this is intense, the specimens are allowed to decay before handling. Twenty minutes usually suffices for this.

For runs involving a variation of ion density the samples are covered with aluminium foils of varying air equivalent. It is necessary that the beam energy be accurately known so that the effective particle energy in the sample can be calculated.

For observations at different temperatures either a liquid cooled sample holder or an electrically heated holder is used. Leads are provided for thermocouples to read the temperature.

The uniformity of the beam is checked by placing paper in the position of the sample and causing it to be blackened by the beam. This also enables the effective area to be measured. With a long tube and a scattering foil, the uniformity is good, though much beam has to be sacrificed to secure it.

The samples are dried in less than milligram amounts on to glass coverslips, or stainless steel discs. These are either held by "Vaseline" or by alnico magnets on to the bombardment disc. Control samples are always treated in exactly the same way as the irradiated ones, including mounting on the bombardment disc in the irradiation chamber.

For electron irradiations the technique is almost exactly the same, with only minor changes in the geometrical arrangement to take into account the greater scattering encountered with electrons.

## Basic Theory and Results of Irradiation Studies

There are at least three broad steps involved in the direct action of radiation, namely (1) the primary ionizations and excitations along the path of a charged particle, (2) the secondary processes by which this energy is converted to broken chemical bonds, and (3) the biological change observed. A great deal is known about (1), a little about (2), and almost nothing about (3) except that it exists. A purely empirical approach allows us to ignore the intermediate steps and apply a statistical analysis to the correlation of the known spatial distribution of primary events to the observed biological end result. The observations which do not fit this "first order" treatment suggest further experiments which may shed light on the intermediate steps. Several of these will be discussed later. At this point we shall outline the first order empirical calculations.

Assume, as Lea did, that one ionization is required to inactivate a molecule. Excitations are assumed ineffective. If $dD$ particles per unit area are fired through an enzyme sample, the chance of a particle passing through a molecule of cross sectional area $S_0$ is $S_0 dD$. Then if

we let $p$ be the probability of one or more ionizations in the molecule if an incident particle passes through it, we find for the chance of inactivation

$$-dn/n = pS_0 dD = SdD \qquad \ldots.(2)$$

where

$$pS_0 = S.$$

Integration yields

$$\frac{n}{n_0} = e^{-SD} \qquad \ldots.(3)$$

$n/n_0$ is the relative biological activity remaining after a total dose of $D$ particles per cm$^2$. The quantity $S$, known as the inactivation cross section, represents the chance of inactivation per incident particle.

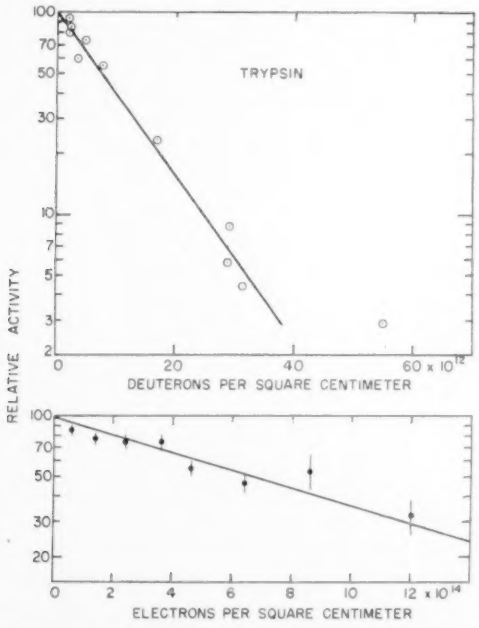


Fig. 2. The relative enzymatic activity of trypsin, on a logarithmic scale, versus the number of incident particles per cm$^2$.[38] The upper curve is for 3·8 Mev deuterons, the lower for 2 Mev electrons. The number of incident electrons is much greater than the number of incident deuterons. Both experiments indicate exponential inactivation of the type given by Eqs. (1, 3).

Unless the incident particles ionize very densely the inactivation cross section, $S$, will be less than the geometrical cross section, $S_0$, of the molecules. The exponential inactivation expression (3) is typical of all random inactivation processes.

Fig. 2 shows the inactivation curves for trypsin.[38] When $n/n_0 =$

$0{\cdot}37 = e^{-1}, S = 1/D_{37}$. We see that $D_{37} = 10 \times 10^{12}$ deuteron/cm$^2$ and $S = 10^{-13}$ cm$^2$. In this case the inactivation cross section is much smaller than $S_0$ because of the sparse nature of the ionization, which averaged about 2 primary ionizations per 100 Å of protein.

The case of inactivation by medium energy protons or deuterons, where the events are localized along tracks but at intervals of the order of magnitude of the molecular dimensions, has been attacked from two points of view. LEA[9] allows the volumes associated with the primary ionizations to overlap, thus putting more than one event in the same space and wasting some of them. POLLARD and FORRO[11] on the other hand proceed in a manner implied in Eq. (2) and calculate the probability, $p$, of at least one ionization occurring in the thickness of the molecule. If the mean number of primary ionizations per centimeter is $i$ and the average thickness of the molecule $t$, the mean number per molecular thickness is $it$. The Poisson distribution gives the chance of *no* ionization $p_0 = e^{-it}$, and thus $p = (1 - e^{-it})$ is the chance of *at least one* occurring. Therefore, $S = pS_0$ becomes

$$S = S_0(1 - e^{-it}) \qquad \ldots.(4)$$

Since the linear ionization density depends on the particle velocity, it is possible to measure $S$ as a function of $i$. The quantity $i$ can be calculated from the experimentally determined rate of energy loss, assuming an average of 110 ev expended per primary ionization.[11]* A few typical values are given in Table I. Extrapolation to high ion density yields $S_0$, which is a valid cross section area and is independent of the shape assumed for the molecule.

TABLE I

*Rate of Energy Loss and Primary Ionizations for Different Particles*[10]

| *Particle* | *Energy* | *Energy loss in protein, ev*/100 Å | *Primary ions per* 100 Å *protein** |
|---|---|---|---|
| Electron | 2 Mev | 2·75 | 0·025 |
| Deuteron | 4 Mev | 215 | 1·91 |
| | 1 Mev | 540 | 4·91 |
| Alpha Particle | 10 Mev | 662 | 6·02 |
| | 1 Mev | 2520 | 22·9 |

* Assuming 110 ev per primary ionization.

* Ref. 11 gives 115 ev/primary ionization, but it is now thought that 110 is a better value.

For fast electron irradiation, on the other hand, $it$ is very small and expansion of the exponential in expression (4) leads to

$$S = S_0 t \,.\, i = V \,.\, i \qquad \ldots.(5)$$

$S_0 t$ is the volume $V$ of the molecule. If we multiply both sides of (5) by $D$, the dose in particles/cm$^2$, we get $SD = ViD = VI$, demonstrating the equivalence of equations (1) and (3) for this case. From (5) we also see that the slope of the $S$ vs. $i$ plot at the origin equals the volume, and electron irradiation results may be used as a check on the shape of the $S$ vs. $i$ curve. Fig. 3 shows such a plot for the enzyme

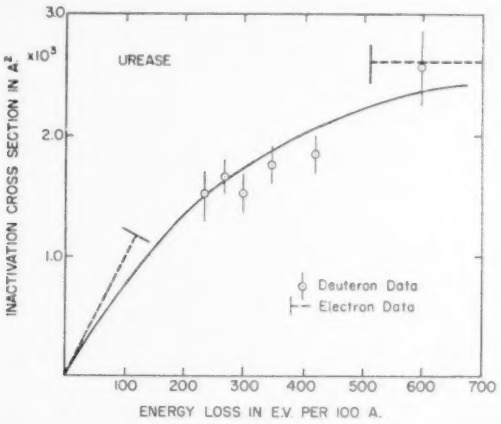


Fig. 3. The inactivation cross section for urease versus the energy loss per 100 Å of the incident particles.[12, 13] The solid curve represents Eq. (4) for a spherical molecule of radius 30 Å.

urease.[12, 13] The experimental points scatter about a curve of the form of Eq. (4) which extrapolates to a limiting value $S_0$ of 2800 Å$^2$. The inactivation volume as found by electron bombardment (see Eq. 1) is 120,000 Å$^3$. The slope at the origin, as determined by electron irradiation, is indicated by the dotted line.

If the molecule is assumed to be spherical, the cross section $S_0$ should yield a volume equal to that derived from the electron data above. In this case we see that the spherical volume corresponding to $S_0$ is 120,000 Å$^3$ compared to the same value obtained from electron data. The close agreement between electron and deuteron inactivation data indicates the basic soundness of LEA's assumptions and the validity of this method of study. Urease clearly acts as if it is a spherical molecule.

Charged particle inactivation data can yield information about the shape of the irradiated molecules. For example, the results of irradiating hyaluronic acid with electrons, deuterons and alpha particles are

TABLE II

*Molecular Sizes Determined by Ionizing Radiation Studies*

(All numbers represent "molecular weights," obtained at room temperature, except where noted)

| *Material* | *Data from:* *Electrons* | *Heavy Particles* | *Reported Size** | *Remarks* | *Reference* |
|---|---|---|---|---|---|
| Penicillin | — | 550 | 356 | Cluster size comparable to molecule | 61 |
| Dysentery toxin | 11,000 | 11,000 | — | Toxic unit | 62 |
| Catalase | — | 58,000 at 90°K | 250,000 | $\frac{1}{4}$, $\frac{1}{2}$, 1, and 2 molecules? | 48, 52 |
| | 110,000 | 110,000 at 300°K | | | |
| | — | 250,000 at 350°K | | | |
| | — | 500,000 at 385°K | | | |
| Urease | 87,000 | 87,000 | 480,000<br>100,000[63] | See HUBER's data below | 12 |
| Invertase | 120,000 | 123,000 | 120,000[47] | Varies with temperature | 47 |
| Pepsin | — | 39,000 | 36,000–39,000 | | 38 |
| Chymotrypsin | 50,000 | 48,000 | 23,000 | Casein digestion assay | 10, 40 |
| | | 28,000 | | Milk clotting assay | |
| Insulin | 23,000 | 23,000 | nx6000 | Assayed for biological activity | 64 |
| Cytochrome oxidase | 40,000 | — | — | See page 105 | 55 |
| Succinic dehydrogenase | 80,000 | — | — | See page 105 | 55 |
| Trypsin | 34,000 | 31,000 | 15,000–24,000 | Dimer? Independent of substrate used for assay | 38, 39 |
| Soybean-Trypsin Inhibitor (STI) | 12,000 | 12,000 | 24,000[54]<br>9,000[74] | Independent of substrate used for assay | 39 |
| Trypsin-STI complex | 30,000 | 30,000 | 41,000[66]<br>30,000[74] | See page 100 | 39<br>65 |
| DNA-ase | 62,000 | 62,000 | 63,000[71] | | |



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TABLE II (*contd.*)

| | | | | | |
|---|---|---|---|---|---|
| Hyaluronidase (HUA-ase) | 75,000 | 75,000 (globular) | 65,000 crude 11,000 purified[73] | See HUBER's data below | 14 |
| Hyaluronic acid (HUA) | 100,000 | 100,000 (long, thin) | — | Same at 90°K | 14 |
| HUA-HUA-ase complex | — | 175,000 | — | Assayed for activity of enzyme | 14 |
| Sheep mucoprotein | — | 82,000 | 87,000[73] | Assumed long and thin | 14 |
| DNA (pneumococcus transforming principle) | $6{\cdot}5 \times 10^6$ | (45 × 3800 Å) | $4\text{–}7 \times 10^6$ [16] | Biologically active unit | 15 |
| DNA (digestion assay) | 2100 | 500 Å² at 3·7 Mev | 8 nucleotides = 2400? 1300 and 5500 found as breakdown products of digest | Assayed for ability to be digested by DNA-ase | 67 |
| DNA (gelation assay) | 200,000 | 200,000 (if long and thin) | | Gel not stable in water | 37 |
| Alpha amylase (*B. subtilis*) | | 145,000 | 100–200,000.[35] Other amylases vary from 45,000 to 152,000 | Multiple ionization requirement (see page 91). See HUBER's data below | 35 |
| Hemoglobin *p*H 5 | 46,000 | 46,000 | 67,000 | | 34 |
| Hemoglobin *p*H 7 | | 66,000‡ | 67,000 | Multiple ionization requirement (see page 91). Effect on solubility of molecule | 34, 42 |
| Bovine serum albumin (bulk) | | 83,000 | 69,000 | Multiple ionization requirement (see page 91). Effect on solubility of molecule | 33 |
| (monolayer) | | 7000 | | Serological unit | 41 |
| Hemocyanin (*helix pomatia*) | | 6,700,000 (alphas) | $6{\cdot}7\text{–}8{\cdot}9 \times 10^6$ | Alpha particles, in solution | 4 |
| Ribonuclease | 30,000 (x-rays) | | 13,000 | | 8 |
| Myosin | 470,000 | | 840,000 | | 8 |
| Di- and tri peptides† | | 400–900 | 132–251 | Assayed chromatographically | 69 |

TABLE II (*contd.*)

The following results are taken from HUBER *et al.*[68], [72] and have been converted to molecular weights by POLLARD,[10] and the authors. Irradiation was by pulses of electrons from a 3 Mev capacitron.

| *Material* | *Data from:* *Electrons* | *Heavy Particles* | *Reported Size** | *Remarks* | *Reference* |
|---|---|---|---|---|---|
| Hyaluronidase | 440,000 | | 65,000[73] | | 68, 10 |
| Diastase | 86,000 | | | | 68, 10 |
| Amylase | 70,000 | | 45,000–152,000 | One datum, 94·5 per cent survival at $10^6$ rep | 68, 10 |
| Lipase | 132,000 | | | 86 per cent survival at $1{\cdot}5 \times 10^6$ rep | 68, 10 |
| Urease | 62,000 | | 480,000<br>100,000[63] | 90·6 per cent survival at $2 \times 10^6$ rep | 68, 10 |
| Cytochrome oxidase<br>Papain<br>Trypsin<br>Pepsin | no detected loss of activity for $3 \times 10^6$ rep | | | Would expect about 10 per cent inactivation at this dose for m.w. 40,000 | 68 |
| Diptheria antitoxin (horse) | 450,000 | Frozen at −35°C | 165,000 | | 70 |
| (rabbit, man) | <40,000 | | 157,000 | Calculation assumes that no effect reported implies less than 20 per cent inactivation | 70 |
| Antipneumococcal Type I (horse) | <80,000 | | 910,000 | | 70 |
| (rabbit) | <80,000 | | 157,000 | | 70 |

* Reported sizes are those quoted by EDSALL[53] except where noted.

† Alanyl-asparagine, alanyl-methionine, carnosine ($\beta$-alanyl-histidine), glycyl-glycine, glycyl-tyrosine, glycyl-phenylalanine, glycyl tryptophane, glycyl-glycyl-glycine. The calculations are based on primary ionization, which overestimates the m.w. for small molecules. See similar result with penicillin.

‡ APPLEYARD[42] quotes 18,000 and 33,000 for electrons and deuterons respectively. On the basis of requiring 3 ionizations in the molecule, the authors calculate 66,000 from the combined data.

shown in Fig. 4.[14] While the slope at the origin obtained from electron bombardment agrees well with the other data, the hyaluronic acid molecule cannot be considered as spherical. The surface to volume ratio is too large for a sphere. If we assume the molecule to be long and thin, as is indicated by conventional means of study, the data may be fitted by assuming the molecule has a diameter 10 Å and a length 1750 Å. Table II presents a summary of much of the available data.

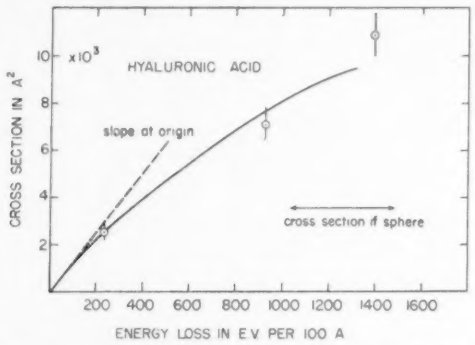


Fig. 4. The cross section as a function of the linear rate of energy loss for hyaluronic acid.[14] The solid curve represents Eq. (4) for a cylindrical molecule of diameter 10 Å and length 1750 Å. The volume of the molecule, from electron irradiation data, is 136,000 Å³. No correction has been made for delta rays.

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Nearly all of the materials listed have shown the following characteristics upon irradiation:

(1) The dose-survival curve is logarithmic: Eq. (1) or its equivalent holds.

(2) The slope of the dose-survival curve is independent of rate of delivery of dose.

(3) The slope is a function of the linear ionization density of the bombarding particle, increasing if dose is expressed as particles/cm².

(4) There is a strong correlation between the slope and the physical size of the target material where the latter is known.

Detailed calculations based on the theory outlined above indicate agreement between the sensitive volume and the known molecular weight to within a factor of about two for a wide range of materials from penicillin (about 350) to the desoxyribonucleic acid (DNA) of the pneumococcus transforming principle (about $6 \times 10^6$). In some cases the factor of two can be reduced by obvious corrections to the first order theory; i.e., penicillin is too small for one to assume the ion cluster confined to one molecule. In other cases, the difference can be correlated with known or suspected structural features of the molecule.

These results indicate that the essential basis of this type of reasoning has been reasonably confirmed and that the basic assumptions are not

gravely in error. One ion pair in a molecule does inactivate it biologically as a general rule, and the effect of primary excitations is at most small.

We conclude that this method may be used to measure molecular weights which cannot be measured in any other way. No great accuracy can be claimed for the method. There are a number of complicating factors which will be discussed in detail in the following sections, and a few molecules have been found which do not fit the simple theory at all. Disregarding all such factors, it is apparent from Table II that orders of magnitude can certainly be relied on, and frequently much better data can be obtained. The salient advantage of the method is that there is no need for purity of preparation. The only requirement is that there be a method for assaying the biologic activity of the molecule, at least semi-quantitatively. At present it is convenient if the preparation is one that can be dried. In practice, this has proven less restrictive than might be imagined, since a wide variety of preparations may be lyophilized. In addition, it is possible that other methods such as irradiating in the frozen state might give equivalent results.

The molecular weights of several of the molecules in Table II have not been measured in any other way. Pneumococcus DNA is an example of a molecule whose molecular weight was measured by radiation techniques[15] and later confirmed by other work.[16] The use of these techniques in the study of virus structure has been discussed elsewhere.[17]

## Spatial Distribution of Ionization

A basic assumption of this treatment has been that one knows the space distribution of ionizing events. This can be determined from a knowledge of the type, energy, and number of charged particles. The rate of energy loss may be calculated from the theories of Bethe[18] and Bloch,[19] which both lead to the expression

$$-\frac{dE}{dx} = \frac{4\pi e^4 z^2}{mv^2} NZB \qquad \ldots.(6)$$

where $dE/dx$ is the rate of energy loss with distance, $x$, in ergs/cm, $e$ the electronic charge, $z$ the number of charges on the particle, $v$ the velocity of the particle, $m$ the rest mass of an electron, $N$ the number of atoms per cm$^3$ of atomic number $Z$, and $B$ a term which for heavy particles is

$$B = \ln \frac{2mv^2}{I} - \ln(1 - \beta^2) - \beta^2,$$

and for electrons is

$$B = \tfrac{1}{2}\left\{ \ln\left(\frac{mc^2\beta^2 E}{2I^2}\right) - \ln(1 - \beta^2) + 1 - \beta^2 \right\}.$$

Here, $\beta$ is the ratio of the particle velocity to the velocity of light, $E$ is the particle energy, and $I$ is a mean excitation potential for the stopping atoms. These equations, on integration, yield range vs. energy relationships which are very well checked by experiment in gases, solids, and liquids with the possible exception of liquid water.[20], [21] There seems to be little question that Eq. (6) holds for dry biological solids.

The next step is to take account of the fact that this energy loss is in discrete steps, randomly distributed along the path of the particle. In particular, we need to know the mean spacing of the primary energy loss events.

One can approach this task from two aspects, the theoretical and the experimental. The relative probability of various size ionizations and excitations can be estimated for atomic *hydrogen* from theory. The number of primary ions per centimeter of path in various gases can be measured directly for fast electrons, by counting the droplets formed on immediate expansion of a cloud chamber after the passage of an electron. Such experiments have been carried out by WILSON,[22] WILLIAMS and TERROUX,[23] and others. POLLARD and FORRO[11] and SLATER[24] have reviewed the data and conclude that for air, neon, argon, nitrogen, and oxygen, the energy loss per cm of path, as given by Eq. (6), divided by the number of primary ions per unit path, gives $110 \pm 10$ *electron volts/primary ion pair*, for high energy electrons and mesons.

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Since, to a first approximation, all processes are affected equally by changing the velocity of the incident particle, and the mass of the particle is not important, the same figures should be very nearly applicable to deuterons and alpha particles. There is still, however, the question of whether these numbers, obtained from electrons in gases, really do apply to all charged particles in solids.

SLATER,[24] points out that the biological irradiation experiments can provide a direct measure of the energy loss per primary ionization. For this method to work, one needs both deuteron and electron inactivation data for a spherical molecule. In this case, then, by extrapolating the deuteron cross section curve to very high ion density, one gets an area measurement independent of any detailed knowledge of the exact ion densities involved. For a sphere, one may then calculate its volume, and knowing this number, solve the electron volume equation backwards; that is, the usual calculation is

$$V = \frac{\text{ev/p.i.}}{\text{ev/cm.electron}} \cdot \frac{1}{\text{electron/cm}^2} \left[\frac{\text{cm}^3}{\text{p.i.}}\right]$$

Knowing $V$ from the deuteron curve, this equation can yield ev/primary ionization for electrons. Furthermore, if one knows the size of a spherical target, the variable in fitting the cross section vs. ion density curve to Eq. (4) is again the energy loss per primary ionization *for*

*deuterons.* Thus a check is available on the experimental data in gases. Slater tried this method for a number of the molecules listed in Table II, with the result that within present experimental error, the number is the same for electrons and deuterons and is again 110 ± 10 ev/primary ionization in apparent agreement with the gas value.

The basic calculation of the linear ionization density therefore rests on the rate of energy loss as given in Eq. (6), thoroughly verified by experiment, and the experimentally determined average energy loss per primary ionization. It should be pointed out that this latter number is averaged over a wide range of values, includes the loss of primary excitations, and does not necessarily represent the energy actually released at the site of the primary ionization. Since about half of all primaries have no associated secondary ionizations, it is probable that less than 30 ev is actually released at the site of these, and is divided between the ejected electron and the ionized atom.

It has been assumed so far in the discussion that the ionizations produced are directly in the path of the ionizing particle. This can be shown to be reasonably true of the primary events caused directly by the ionizing particle. (See POLLARD and FORRO.)[11]

An incident ionizing particle will produce recoil electrons, called delta rays, having energies from thermal up to a maximum, depending on the mass of the particle. An incident electron can transfer half its energy, whereas a 4 Mev deuteron can transfer a maximum of 4350 ev. Delta rays having energies less than about 100 ev will not go more than a very few atomic diameters before losing nearly all their energy, and the secondary ions produced by such a delta ray may be considered as a small cluster. Electrons of higher energy can travel a considerable distance, over 4000 Å for a 4000 volt electron, and thus produce secondary ionizations at a considerable distance from the track. About 30 per cent of the total energy dissipated by a 4 Mev deuteron is transferred to electrons of energy greater than 100 ev, so that the problem is serious if precision studies are being made.

LEA[9] made a substantial contribution towards calculating the effects of these delta rays with his "associated volume" concept. Since Lea calculated the tables used for the associated volume calculation, additional factors have come to light which indicate that his figures seriously overestimate the delta ray corrections. These are:

1. Ranges of slow electrons in proteins have recently been measured by DAVIS.[25] The data show ranges for electrons of energies 100–1000 ev which average less than half the calculated ranges used by Lea.

2. It was assumed that the delta rays went in straight lines. Actually, electrons having such energies are very strongly scattered, and on the average the paths are considerably curled up.

3. The assumption that the delta rays go off at right angles to the track of the particle is not valid. Actually, the initial angle of projection $\theta$ with the track is given by[18]

$$\cos^2\theta = \frac{\text{delta ray energy}}{\text{maximum delta ray energy}}$$

This shows that the higher the energy of the delta the more accurately it is projected down the track of the primary particle where it does not contribute more biological effect. It will soon get scattered out of the track, but it should be noted that the scattering is least at the highest energies.[26]

These factors reduce the effectiveness of delta rays. It seems likely that LEA's associated volume calculation overestimates the effect by at least a factor of two, and probably more. In the absence of a reliable theory, the delta ray correction has been ignored in practically all the data which have been reported. Perhaps an indication of the magnitude of the correction is the amount of information which has been obtained without it. A good theory, however, would be of great help in putting the data on a firmer basis.

Except for the delta ray correction, then, the calculation of the space distribution of ionizations is based on well tested theory and experiment.

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## ENERGY REQUIREMENTS FOR INACTIVATION

The very good general agreement between the simple first order theory and the data in Table II is impressive evidence for LEA's hypothesis that a single ionization will inactivate a molecule. Many of the primary ion clusters consist of several ions, but about half of the primary clusters contain only 1 ion (see Table III), and the weight of the evidence seems to indicate that even this single event will cause inactivation.

The probability that an excitation will inactivate is much less clear. The relative probability of a charged particle losing a certain amount of energy has been measured directly by RUTHEMANN[27] and by LANG.[28] They passed a monochromatic electron beam of energy about 5 kv through very thin ($\sim$ 100 Å) foils of various metals and collodion and analyzed the emerging electrons in a 180° spectrometer. Some of the foils were thin enough so that the probability of an electron making more than one inelastic collision was small. The data show that the probability of an electron losing the order of 5 ev is small, and the most probable energy loss is the order of 25 ev. From these data it would be anticipated that very few primary excitations would be created by an incident particle. The excitations which are created are thus almost entirely confined to the vicinity of the ion clusters formed when a sufficient amount of energy is transferred to an atomic electron.

Thus it would appear to make little difference to the simple theory whether or not an excitation inactivates.

It should be pointed out that calculations on atomic hydrogen indicate approximately 6 primary excitations for each primary ionization.[18] It may be that atomic hydrogen is sufficiently atypical to account for the difference between the calculation and the experiment quoted above.

There are other reasons for suspecting that an excitation is unlikely to cause inactivation. A most important one is the low quantum

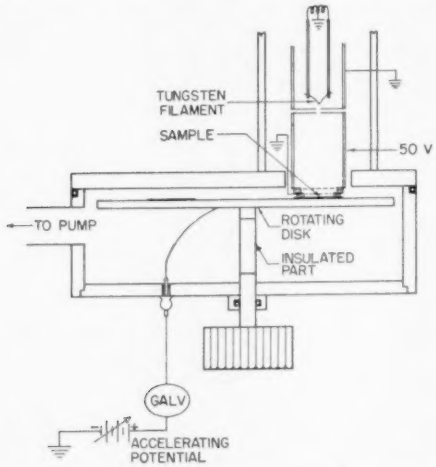


Fig. 5. Experimental arrangement for slow electron irradiation.[30]

efficiency for ultra-violet inactivation in the wavelength range 2000–3000 Å.[29]

A direct test of the energy required to produce an inactivation has been measured for the case of monomolecular layers of bovine serum albumin, using a beam of very low energy electrons.[30] The assay used to determine when the molecules had lost their biological activity was the ability of the molecule to combine with the homologous rabbit antibody.

The apparatus used is shown schematically in Fig. 5. The effective electron beam energy could be varied from 2 ev to 250 ev. The albumin molecules were laid on a chromium surface, and only half of each sample irradiated, the other half being kept as a control. A semi-log plot of the surviving activity against dose in electrons/cm$^2$ gave somewhat curved lines, concave upwards, so that the initial slope of the curve for a given energy electron beam was taken as a measure of the effectiveness and converted into a cross section. Fig. 6 shows a plot of these cross sections against accelerating voltage.

It is seen that from 2 to 10 volts the cross section is small. The processes occurring are probably similar to those involved in ultraviolet inactivation. The lowest energy at which measurements could be made, 2 ev, corresponds to a 6000 Å photon, in the visible. However, an electron approaching a dielectric surface such as a protein experiences an attraction due to "image" forces. The magnitude of the additional energy which an electron gains from this can be estimated

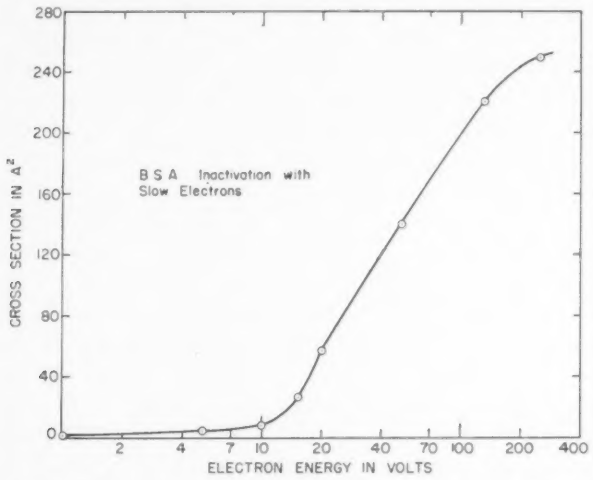


Fig. 6. The cross section for the inactivation of the antibody combining power of bovine serum albumin versus the energy of the incident electrons.[30] Note the rapid rise in cross section for energies which can cause ionization. The abcissa is actually the applied accelerating voltage, and the mean electron energy is one volt greater (thermal energy, etc.).

to be the order of a few ev, which is sufficient to bring the observed inactivation into agreement with the ultraviolet data.

Above ten volts the cross section increases very rapidly with energy. Ten ev is about the expected value for the ionization potential of a protein, judging from data on hydrocarbons.[31] The variation of the cross section with voltage is very similar to that found for the cross section for ionization for gas molecules.[26] It is natural to ascribe the increase to the process of ionization, and thus by inference assign to it the major role in the action of high energy radiation on biological material.

This experiment gives one more piece of information about the inactivation process. Granting that the inactivation is caused by an ionization, the question now comes up as to whether it is bond breakage due to the absence of an electron, the energy released when an electron is recaptured, or some other similar process. A possible inactivation mechanism is simply the electric field produced by a free electron



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released into the protein lattice by ionization. This hypothesis does not fit in with Fig. 6. For incident electrons of energy $\sim 30$ ev the likely number of electrons released could not exceed twice the incident number, yet the cross section increase is much greater than this. Thus the effect of this process, if any, must be small.

An interesting point is that the maximum cross section observed with the electrons is in good agreement with the maximum cross section estimated in the same way for high energy deuteron inactivation of serum albumin monolayers.[32] The agreement between the two cross sections found for widely different types of radiation is a strong argument for believing that the sensitive volume corresponds to an actual physical unit, and is not merely a convenient parameter for classifying the effect of radiation on a substance.

### Multiple Ionization

An experimental fact of great importance is that the fraction of biological activity of a protein surviving a given amount of radiation depends exponentially on the dose. No sign of curved lines, concave downwards, on a semi-log plot, indicating either the need for hits on the same target molecule by several particles, or hits on different target areas in the same molecule, have ever been observed in dry irradiations of proteins with ionizing radiation. We conclude that the evidence is in favour of the hypothesis that the passage of a single charged particle is sufficient to inactivate a single biological molecule. This does *not* imply that every particle that passes through the molecule, or even releases energy in the molecule, necessarily inactivates; only that the actual event which causes the molecule to be classed as an inactive one is due entirely to the effects of a single particle.

This is in agreement with the simple theory given above. The simple theory also demands that the shape of the curve of cross section against rate of energy loss be that shown in Figs. 3 and 4. There is always the possibility that a molecule is inactivated by a single ionization, but that only a certain fraction $f$ of the primary ionizations within the target volume result in permanent damage, the remaining ionizations producing no lasting effects. Under this assumption Eq. (4) becomes

$$S = S_0(1 - e^{-fit}) \qquad \ldots.(7)$$

The experimentally measured target cross section with densely ionizing particles will be the full cross section, the shape of a plot of cross section vs. rate of energy loss will be the same, but the apparent thickness of the molecule will be only the fraction $f$ of the actual thickness, and the volume measured with electrons will be $f$ times the actual.

The most convincing reason for believing that in most cases all of the ionizations in a given molecule are effective is found in the general

agreement between the target volumes determined from electron irradiations and the molecular weights of the molecules irradiated as measured by such totally different methods as sedimentation-diffusion. (See Table II.) We conclude that for most of the substances which have been studied the fraction $f$ of effective ionizations must be very close to one.

There are at least three proteins, bovine serum albumin,[33] hemoglobin,[34] and alpha amylase,[35] which show a dependence of cross

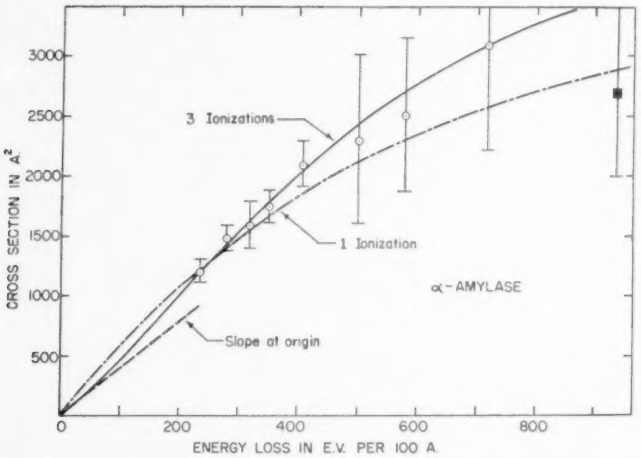


Fig. 7. The cross section for the inactivation of alpha amylase versus the linear rate of energy loss.[35] The slope at the origin is obtained from electron inactivation data. The 1 ionization curve represents Eq. (4) for a spherical molecule of 18 Å diameter and 275 Å length, and the 3 ionization curve represents Eq. (10) for a spherical molecule of 183,000 Å³ volume.

section on rate of energy loss which is definitely not in agreement with Eq. (4). (See Fig. 7.) Qualitatively, the effect appears to be an increasing effectiveness of a primary ionization as the average separation of the primary ionizations decrease.

An obvious hypothesis is to assume that *two primary* ionizations within the target volume are necessary to cause inactivation. This can be readily disproved by noting that the predicated sensitivity to electrons would be very small, because of the unlikelihood of two primary ionizations, with a mean separation of 4400 Å along the electron track, falling into a single molecule of dimensions the order of 50 Å. Experimentally the electron sensitivity is less than would be expected, but it is not nearly as small as the hypothesis above would predict.

The primary ionizations which are created along the path of the ionizing particle are not all the same. In some the ejected electron does not have sufficient energy to create further ionization, in other cases



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the secondary electron is able to ionize another nearby atom, and in still others the secondary creates two, three, or even more subsidiary ionizations clustered closely about the primary event. Table III gives some figures for the relative probability of these ion clusters containing various numbers of ions, as determined from experiments with electrons in cloud chambers.

TABLE III

*Percentage of Primary Ion Clusters having Various Numbers of Ions*

| *Number ions in cluster* | *Air* | | *Neon* | *Selected number* |
|---|---|---|---|---|
| | Beekman[36] | Wilson[22] | Beekman[36] | |
| 1 | 61 | 42·5 | 50 | 50 |
| 2 | 21·5 | 22·5 | 22·5 | 25 |
| 3 | 8·5 | 12·5 | 10·5 | 10 |
| 4 | 4 | 10 | 7 | 5 |
| > 4 | 5 | 12·5 | 9·5 | 10 |

The hypothesis can be made that the important parameter in determining whether or not a protein is inactivated is the total number of ionizations simultaneously produced within the target volume.[33] If the critical number is two, it is explicitly assumed that it makes no difference whether there is a single primary ion cluster containing at least two ions, or two single primary ionizations at opposite ends of the target volume.

The probability for this can be calculated by a method closely related to the derivation of Eq. (4). That equation gives the probability that a molecule of effective thickness $t$ has at least one primary ionization created within it. From this must be subtracted the probability that the molecule contains only a single primary ion cluster which consists of only a single ion. Let the probability that a cluster contains only a single ion be $q_1$. The probability that just one primary ionization occurs in the molecule can be calculated from the Poisson equation for the probability of just $n$ random events occurring, when the average number is expected to be $a$ events, which is

$$P_n = \frac{a^n e^{-a}}{n!} \qquad \ldots.(8)$$

Putting $a = it$, the average number of primary ionizations in a molecule of thickness $t$, and $n = 1$, and multiplying by the probability that the primary ion cluster contains only a single ion, we get $q_1 ite^{-it}$. Subtracting this from Eq. (4) gives the probability, $P_2$, for two or more ionizations in a molecule

$$P_2 = 1 - (1 + q_1 it)e^{-it} \qquad \ldots.(9$$

In much the same way the probability $P_3$ that at least three ionizations are put in the molecule can be calculated. In this case, from Eq. (4) must be subtracted the probabilities that (a) only a single primary ion cluster with less than three ionizations in the cluster occurs, and (b) just two primary ion clusters form, neither of which have two or more ionizations. The probability of (a), by analogy to the two ionization case, is $q_2 ite^{-it}$ where $q_2$ is the fraction of primary ion clusters having less than three ionizations. The probability (b) is the product

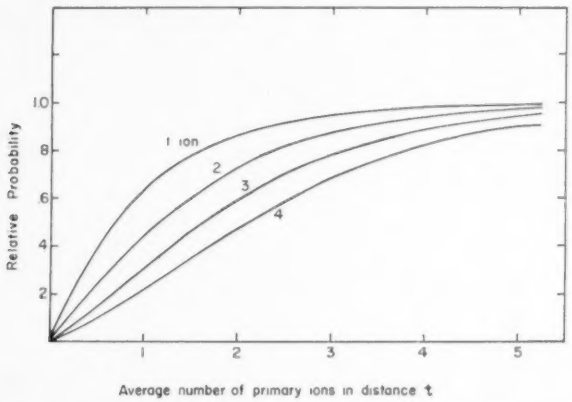


Fig. 8. The probability of finding various numbers of ionizations occurring within a molecule of thickness $t$ as a function of the average number of *primary* ionizations falling within the molecule.

of the probability of just two primary ions, given by Eq. (8) with $n = 2$, and the probability that *neither* of the two ion clusters contains more than one ion, which is $q_1^2$. Thus

$$P_3 = 1 - \left\{ 1 + q_2 it + q_1^2 \frac{(it)^2}{2} \right\} e^{-it} \qquad \ldots\ldots(10)$$

In a similar way the probability of four simultaneous ionizations may be calculated as

$$P_4 = 1 - \left\{ 1 + q_3 it + q_1(2q_2 - q_1) \frac{(it)^2}{2} + q_1^3 \frac{(it)^3}{6} \right\} e^{-it} \qquad \ldots\ldots(11)$$

The quantities $q_1$, $q_2$, and $q_3$ may be determined from the data in Table III to be $q_1 = 0{\cdot}50$, $q_2 = 0{\cdot}75$, $q_3 = 0{\cdot}85$. A plot of these probabilities as a function of the variable $(it)$ is shown in Fig. 8. With the three proteins bovine serum albumin, hemoglobin, and alpha amylase, a good fit can be obtained by assuming that three simultaneous hits are necessary to inactivate them. The appropriate values of

the target thickness and cross section are given in Table IV. It is interesting to note that both of the established cases in which the inactivation is essentially an insolubility effect (bovine serum albumin and hemoglobin) have this multiple ionization requirement. The alpha

TABLE IV

*Parameters for the Inactivation of Proteins Requiring Multiple Ionization*

| *Protein* | *Cross-Section* | *Thickness* | *Molecular Weight* | *Reference* |
|---|---|---|---|---|
| Bovine serum albumin | 3800 Å² | 28 Å | 83,000 | 33 |
| Alpha amylase | 3900 | 47 | 143,000 | 35 |
| Hemoglobin | 2400 | 35 | 66,000 | 34 |
| Cytochrome oxidase | 5600 | 36 | 160,000 | 55 |
| Succinic dehydrogenase | 9400 | 36 | 270,000 | 55 |

amylase preparation was a very impure one, and it was not possible to determine whether or not the inactivation involved the solubility of the enzyme after irradiation.

It is possible that the real requirement for the inactivation of the molecule is not one of ionization, but one of energy released in the molecule. Determining the energy requirement in terms of the ionization produced is rather crude, but a more accurate theory would be quite complex.

## Temperature, *p*H Effects, Energy Migration and Spread

The observed effects of the direct action of radiation on proteins, nucleic acids and the like depend not only on the type of molecule investigated, and on the spatial distribution of the ionizations and excitations produced by the incident radiation, but also on the physical state of the material during irradiation and on its treatment after irradiation. To take an extreme example, it is found[37] that desoxyribonucleic acid when irradiated with fast charged particles is completely soluble in water, but if the same DNA is placed in salt solution after irradiation, it forms a gel. Among the post-radiation factors which may influence the observed effects of radiation are *p*H and ionic strength of the resuspending medium, the temperature of the samples after irradiation, and the method of assay. Physical factors during the irradiation which may alter the results are the temperature and the

interaction between the material under investigation and its surroundings.

*Method of assay.* If the action of radiation is to make an enzyme so unstable that it adopts a random configuration when placed in solution, we would expect that the inactivation cross section of the molecule would be independent of the method of assay. This conclusion would hold even if different sites of an enzyme molecule are concerned with different enzymatic specificities. It is found that the cross section for trypsin inactivation is the same for the ability of trypsin to digest casein,[38] and its ability to hydrolyze benzoyl arginine ethyl ester.[39] On the other hand, the cross section for chymotrypsin depends upon whether it is assayed by casein hydrolysis or by its milk-clotting activity.[40] The cross section for the latter case corresponds to a molecular weight of 28,000, while for the former it is 48,000. These data have been interpreted as indicating that roughly two chymotrypsin molecules are associated with hydrolysis, while one molecule is concerned with the clotting of milk.

Bovine serum albumin has been assayed by its ability to combine with antibody in solution and when the albumin is in the form of a monolayer. Widely different results are obtained. If it is irradiated dry and in bulk[33] it is found that three simultaneous ionizations anywhere within the entire molecule (molecular weight 69,000) will make it insoluble, and hence unable to react in a serological assay. If the molecule is transferred on to a backing from a monomolecular layer spread on a water surface,[32] or if normal albumin molecules are adsorbed on a surface,[41] both the irradiation and the assay can be done *in situ*, and hence the solubility of the molecule is never involved. Under these conditions the target volume is approximately a sphere 13 Å in radius, with a molecular weight the order of 7000. Undoubtedly in the case of the irradiation of the bulk bovine serum albumin some of the inactivation was due to single ionizations in the small serologically active zone, but the much higher probability of the molecule becoming insoluble and the considerable errors involved in serological assays prevent the two different processes from being apparent in the data on the bulk albumin.

Hemoglobin irradiated by deuterons[34, 42] shows the same cross section when assayed by its solubility at *p*H 7 and by its ability to combine with its homologous antibody. Invertase has been assayed after irradiation for its enzymatic activity and its ability to combine with antibody.[43] The antibody combining power has a smaller cross section, but this may be because the antigen is not the same as the enzymatically active particle. Obviously from all these data we can draw no firm conclusions.

*Evidence for only partial damage.* An inspection of Table II indicates



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that in some cases the radiation molecular weight is less than the value obtained from sedimentation and diffusion studies. In some instances, urease for example, the difference is the fault of the sedimentation and diffusion studies, and not the radiation value. This is because radiation analysis can use impure and very dilute samples, while the more conventional means require high purity and high concentration, which in turn favours polymerization of the fundamental enzymatic unit.

There are, however, radiation molecular weights which are smaller than the accepted values only if the samples are treated in a special manner after irradiation. Thus irradiation of hemoglobin by electrons[34] indicates that the volume damaged corresponds to one quarter of the molecule if the samples are dissolved in a medium of *p*H 7, whereas dissolving in a medium of *p*H 5 gives a figure approximating the entire molecule. It is pertinent that the only difference between these two results is the medium in which the samples were dissolved. The number of ionizations produced by the incident radiation and the amount of energy liberated is the same for both cases. Since, then, a dissolving medium of *p*H 5 gives a much bigger cross section than dissolving at *p*H 7, we are entitled to say that at *p*H 7, those molecules which remain in solution are no longer the same as native hemoglobin molecules.

Another way of demonstrating that an ionization within a molecule changes its structural integrity but does not necessarily cause the loss of enzymatic activity is by heating the molecules after irradiation. (We would only expect to get a positive result in this case if the radiation molecular weight is less than the true molecular weight.) Catalase is a good example of this effect.[44] If it is heated after irradiation, the usually observed exponential decrease in activity is replaced by a curve which can be resolved into the sum of two exponentials; one component has a cross section corresponding to the value obtained with no heating, the other component has a much larger cross section. (See Fig. 9.) The latter component obviously represents the heat inactivation of damaged but enzymatically active molecules. This heat inactivation takes place at a high rate compared to the heat inactivation of normal catalase.

The effects of *p*H on the radiation sensitivity of trypsin[45] may also be explained by this partial damage hypothesis, although in the latter case the *p*H effect may be the result of different states of aggregation of the trypsin during the irradiation in solution. By far the best example of partial damage to a molecule is the experiments on the inactivation of monolayers of bovine serum albumin.[32] The antigenic cross section observed in this case represents only about $\frac{1}{10}$ the volume of the molecule. Obviously the molecule as a whole is very severely damaged before anything happens to its ability to combine with antibody.

*Temperature during irradiation.* Surprisingly enough it has been found[4, 46] that the temperature of samples during irradiation may have a profound influence on the observed radiation cross sections. It might have been expected that the small variations in thermal energy available to the experimenter would have a negligible effect on a process which is the result of the liberation of energies above 10 ev.

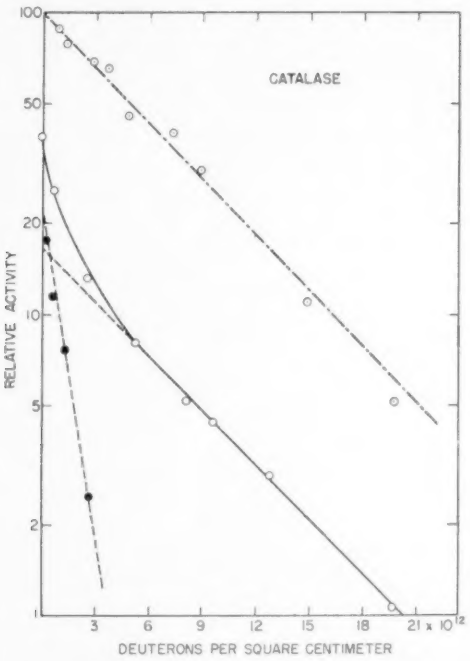


Fig. 9. The relative activity of catalase versus the number of incident 3·8 Mev deuterons per cm².[44]
——— unheated samples.
———— heated to 40 per cent activity *before* irradiation.
— — — — resolution of "heated" curve into two components.

This temperature effect has nothing to do with the migration of chemical groupings or active radicals from one place to another, since in the work reported here the samples were irradiated in a high vacuum. The two proteins which have been investigated most thoroughly with regard to temperature during irradiation are invertase[47] and catalase.[48] The temperature dependence for invertase is found if it is irradiated by electrons, deuterons, or alpha particles and so the temperature effect is associated with charged particle radiation in general. The inactivation cross section of catalase is shown as a function of



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temperature in Fig. 10. These results have been interpreted in terms of the known properties of catalase to indicate that at low temperatures one quarter of the molecule is inactivated, that at intermediate temperatures one half the molecule is inactivated, and that at high temperatures, less than 370°K, the whole molecule is inactivated by a primary ionization. At still higher temperatures, both catalase and invertase show a cross section well above that of the molecule. At these temperatures a particle passing outside the molecule inactivates it.

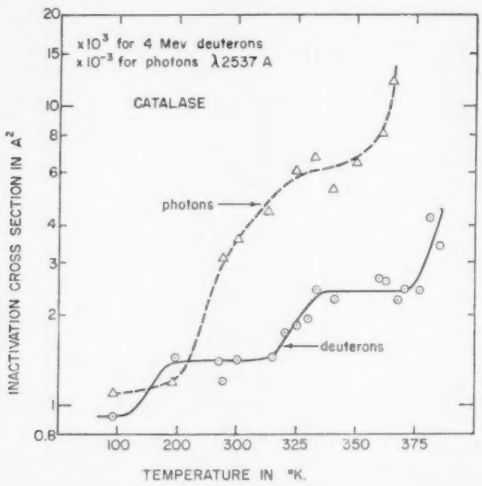


Fig. 10. The inactivation cross section of catalase as a function of temperature for deuteron[48] and photon[49] inactivation. The temperature scale is not uniform.

This effect could arise either from an excitation due to the passing particle, or from an indirect effect on the catalase molecule by surrounding material. Nothing is known about the temperature dependence of such indirect effects. It is known that the ultraviolet sensitivity of proteins increases at high temperatures, and decreases at low temperatures (see Fig. 10). A decrease in sensitivity for ionizing radiation at low temperature has been reported for bovine serum albumin[33], for papain,[50] and for trypsin.[45] In the latter case the irradiation was done in solution, and the extent of the indirect effect is difficult to assess.

At low temperatures the transforming principle for Pneumococcus, a specific nucleic acid polymer, is less sensitive to radiation.[15] The sensitivity of hyaluronic acid does not change appreciably below room temperature.[14]

There exists at present no adequate explanation for the temperature effect of ionizing radiation. It is obviously something that must be

explained by any detailed theory on the action of charged particles on proteins and the like. In qualitative terms, the explanation may be that the decrease in bond lengths at low temperatures is sufficient to impart extra stability to the molecule. Alternatively, we may imagine some hypothetical transfer of energy through the molecule which is very temperature dependent. It is significant that the large increase in invertase and catalase cross sections at high temperatures takes place just before the heat-inactivation rate becomes appreciable.

## Energy Migration

The ability of energy the order of the ionization potential to migrate through a molecule is most clearly shown by the data on hydrocarbons discussed by Wallenstein *et al.*[51] If the members of an isomeric series of aliphatic hydrocarbons such as the octanes are introduced into a mass spectrometer and bombarded with electrons, the ionized fragments which are produced can be studied. It may be safely assumed that the bonds initially ionized by the electrons are distributed at random, but the fragments which appear demonstrate that the bonds are not broken at random, but in a definite pattern. In general, although there appear to be a few exceptions, a bond known to have a low dissociation energy is far more likely to break than a stronger one. As a result isomers with different bond arrangements have widely different mass spectra. This distribution of fragments is not connected with some unusual distribution of the neutral fragments (which are not observed), as is shown by the fact that all isomers have very closely the same total ionization. The only possible conclusion is that energy absorbed in one bond can be transferred to another.

Table II indicates that the radiation molecular weights for some substances are greater than the usually accepted values. While the differences may in part be due to inadequate knowledge as to whether it is the monomer, dimer or higher polymer which is the active enzyme, the values for trypsin and insulin suggest that an ionization in one member of a polymer may very possibly inactivate the other units also. The available data indicate that surrounding impurities do not increase the radiation inactivation of a molecule. Crystalline and non-crystalline trypsin have the same cross section.[38] Non-crystalline catalase is more stable radiation-wise than crystalline catalase.[52] Finally, the activity would not decrease exponentially if a molecule were to be inactivated by energy coming from an already inactivated neighbour, but would show a typical cumulative dose or multi-hit type of effect.

Of considerable interest in this regard, are the results found by Davis[25] in bombarding invertase with low voltage electrons. These experiments were aimed at measuring the ranges of low voltage electrons in proteins and the technique used was to burn off, by intense



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bombardment, as much invertase as any one voltage electron beam could penetrate. The sensitive invertase assay then permits an estimate of the depth of enzyme inactivated. The results are shown in Fig. 11. Up to about 600 volts the depth burned off is constant *and equal to one molecular thickness.* The inference is that ionization in any part of the molecule inactivates that molecule completely, leaving no residual activity whatsoever throughout the molecular volume. This inactivating effect, strong as it is in the top molecules, is *not* passed on to the underlying molecules through the relatively weak Van de Waals forces.

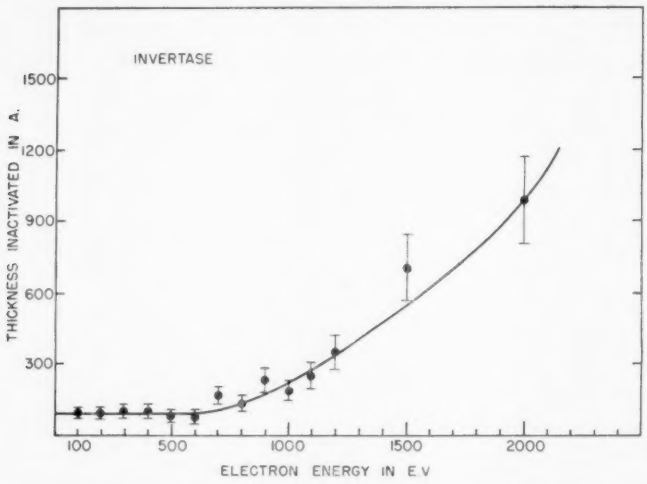


Fig. 11. The amount of invertase inactivated as a function of electron energy.[25]

A direct attack on this question of inactivation of an enzyme molecule by surrounding molecules has been made for two systems; one of these systems is trypsin plus soybean-trypsin-inhibitor. The radiation molecular weight of trypsin is about 34,000.[38] The accepted value is about 17,000.[53] The radiation molecular weight of soybean-trypsin-inhibitor is 12,000.[39] The usually accepted value is 24,000,[54] indicating that the inhibitor either possesses an internal structure, or has an active molecular weight of 12,000*. Trypsin and soybean-trypsin-inhibitor may be dried from a solution at *p*H 5, at which *p*H the trypsin and its inhibitor are known to be combined. After irradiation, the enzyme-inhibitor complex may be dissociated at a low *p*H and the total amount of active enzyme determined. It is found[39] that the enzyme in the complex acts as a spherical target of molecular weight of about 30,000. It is *less sensitive* to radiation than trypsin alone. It

* Recent X-ray crystallographic work by CRICK[74] indicates that the inhibitor's molecular weight is 9,000 and that of the enzyme-inhibitor complex is 30,000.

is tempting to ascribe the figure 30,000 to the combination of one trypsin molecule, 17,000, plus one soybean-trypsin-inhibitor molecule, radiation molecular weight 12,000. The experiment is not as clear cut as it seems, however, because the figure for the complex is within experimental error of the radiation molecular weight of trypsin alone. In the latter case it would be necessary to assume that the trypsin exists in the form of a dimer and is not combined with its inhibitor. This assumption is not in accord with observations made on the complex in solution.

Another system which has been investigated from the point of view of the effect of one molecule on another is that of hyaluronic acid and its specific enzyme hyaluronidase.[14] Bombardment by 4 Mev deuterons indicates that the cross section of hyaluronic acid is 3300 Å$^2$, while that of the enzyme is 1650 Å$^2$. If enzyme and substrate are mixed together at a *p*H at which they react and are then dried rapidly, the cross section for the enzyme is much larger than the figure given above. In some cases it has been found to approach 5000 Å$^2$, a figure which is the sum of the cross sections (or chances of inactivation) of enzyme and substrate. If the enzyme is dried in the presence of digested hyaluronic acid or dried from water, where enzyme and substrate do not react, or dried with a mucoid which it does not attack, then the cross section for enzyme inactivation is the same as for enzyme alone. The results indicate that the specific combination of enzyme and substrate results in the inactivation of the enzyme by an ionization not only in the enzyme molecule but also in the substrate molecule. Whether the enzyme is inactivated by a direct energy transfer from substrate to enzyme or by the formation of a covalent bond between enzyme and substrate (thus inhibiting the enzyme) is not known.

The observed inactivation of a molecule by an ionization in another one to which it is *closely* bound, may represent the explanation for the difference between radiation and accepted molecular weights of insulin, trypsin and chymotrypsin.

## Suggested Physical Picture

The actions of ionizing radiations on molecules are believed to be governed by physical principles which are well understood. Although many features of protein molecular structure remain to be worked out, truly remarkable advances have been made along this line, especially in the past few years. Despite the gaps in our knowledge of proteins and the complexities of applying the concepts of atomic and molecular physics to the situation at hand, the time seems ripe to put down a few speculations about the mechanisms of the action of ionizing radiation. If all they do is to stimulate efforts to disprove them, they will have served their purpose.



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The most important features of radiation action seem to be that (*a*) an ionization has an efficiency for inactivation of the order of one, (*b*) excitations the orders of 4–6 ev have a very small efficiency, (*c*) the efficiency of both ionization and excitation for inactivation increases with temperature, and (*d*) inactivation is usually confined to the molecule in which the energy is released.

The effect of temperature, involving quanta of energy the order of a fraction of an electron volt, on events initiated by ionization involving energies of 10 ev and up indicates strongly the possibility that the breaking of two or more bonds enters into the inactivation process. To form a picture of what is happening, let us first assume that energy released in one part of a molecule can migrate to another, and that molecular fragments formed by impact move away from each other with essentially thermal energies. Both of these assumptions are true for gaseous hydrocarbons ionized by electron impact.[51] We can then visualise excitation energy moving along a polypeptide chain, occasionally giving a valence bond a chance to open. Because of the interlocking hydrogen bonds in a protein molecule, the free ends cannot separate very far, and the bond can be reformed, unless a bond happens to open adjacent to a site where one or more of the hydrogen bonds have been broken by thermal action. At such a site the free ends move away from one another and the highly specific configuration necessary for enzymatic action is lost. The low temperature stability is then attributed to the smaller number of broken hydrogen bonds and the consequent increased chance for the migrating energy to be degraded below that needed for dissociation before arriving in a valence bond adjacent to a large enough number of broken side bonds.

The abrupt increase in radiation sensitivity at a temperature just below the point at which the protein inactivates thermally at a rapid rate must be due to another process. In this region the observed cross section is larger than the cross section of the molecule. A possible explanation is that much of the inactivation observed is actually thermal. The small additional energy needed to inactivate a molecule "thermally" under these circumstances could come from an adjacent molecule which had been hit by an ionization, or from an excitation.

In the case of an inactivation process which does not depend very critically on the specific configuration of the molecule, it is possible that two or more chains must be broken simultaneously to produce the desired result. This provides an explanation of the requirement of multiple ionization for the inactivation (which is principally a loss of solubility) of such proteins as hemoglobin and bovine serum albumin.

It is not clear if the inactivating event is the actual removal of an electron from its normal position, or merely that the excitation energy for inactivation happens to be about the ionization potential. It is

attractive to consider that the decisive event is the removal of an electron. The excess positive charge represents the region of high excitation, which may migrate around the molecule by electrons jumping from bond to bond.

In some cases, the electron must be recaptured by the positive ion, since at low temperatures many ionizations do not result in inactivation. In three cases out of four the recaptured electron will have its spin aligned parallel to the electron with which it is paired, giving rise to a triplet state which will generally have a long de-excitation time. Since half of all primary ionizations are accompanied by one or more secondary ionizations, about 85 per cent of all primary ionizations could give rise to a triplet state. The accuracy of the data in Table II and the knowledge of various parameters such as the number of primary ionizations created by a given radiation are not inconsistent with the assumption that only this 85 per cent of primary ionizations are effective. The triplet state might allow the energy to migrate as a unit for a longer time than would the normal singlet states such as would be excited in an optical transition.

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The picture thus given fits many of the general facts which have been accumulated about the direct action of ionizing radiation on proteins. Many checks on the details of a possible physical interpretation will be necessary before any particular theory can be taken seriously.

## Applications to Enzymes Within the Cell

From the point of view of a biophysicist seeking to use this technique to measure the sizes and shapes of enzymes inside the cell, and so of contributing to knowledge of cellular structure, one point of great importance arises. This is the question as to whether the size determined from radiation action on an enzyme in a cell is the same as that found in purified, or semi-purified preparations. In order to attempt to answer this, bombardments of four enzymes have been undertaken, both as laboratory preparations and in intact cells. These four are invertase in yeast; cytochrome oxidase and succinic dehydrogenase in *B. subtilis*; and amylase in barley seeds. The case of invertase[55] is perhaps the simplest. The inactivation of this in yeast cells is shown in Fig. 12. Deuterons of 4 Mev energy and electrons of 2 Mev energy were used as the bombarding particles. It can be seen that the inactivation line running through the data fits that for commercial preparations indicating that the intracellular enzyme does not differ in sensitivity from the separated enzyme.

Of considerable interest is the bombardment of lyophized *B. subtilis* cells and lysates by electrons and deuterons.[55] The lysates were assayed for cytrochrome oxidase and succinic dehydrogenase by a

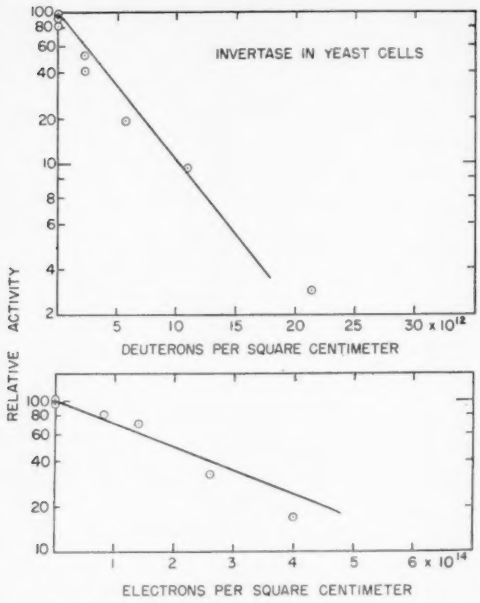


Fig. 12. The relative activity of invertase in dry yeast cells versus the incident dose of 4 Mev deuterons and 2 Mev electrons.[55] The solid lines represent the inactivation curves for the extracted enzyme.

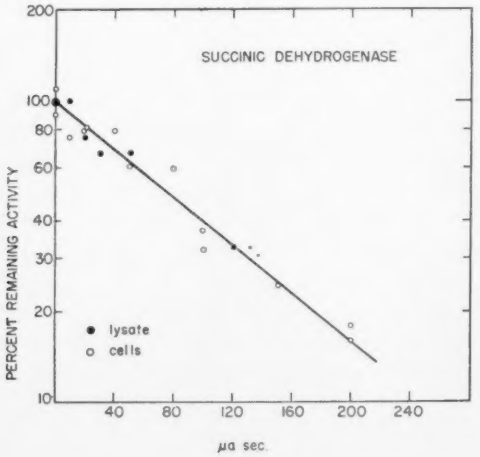


Fig. 13. The activity of succinic dehydrogenase in cells and cell lysates as a function of the dose of 2 Mev electrons.[55] ($100\mu$a. second is equivalent to $4 \times 10^{14}$ electrons/cm$^2$).

spectrophotometric method, and also, for the second enzyme by the reduction of methylene blue. The lysates gave normal enzymatic activities and these were systematically reduced by ionizing radiation action according to the simple relations described. The sensitive volumes for single ionization sensitivity found correspond to molecular weights of 40,000 for cytochrome oxidase and 80,000 for succinic dehydrogenase. When irradiation was carried out on the intact cells, inactivation curves which were substantially the same were found. The curves obtained for electron bombardment of succinic dehydrogenase are shown in Fig. 13. It can be seen that no real difference exists between the two and it can be concluded therefore that for these two enzymes there is no special change in radiation sensitivity in the normal cell or in a lysed preparation. It is of interest that the cross section values obtained from lysate preparations showed a sigmoid tendency when plotted against ionization density. The data are not very accurate but suggest that three ion pairs are necessary for inactivation of these two respiratory enzymes. If this is accepted, the radiation molecular weights are increased above those shown in Table II to give 160,000 and 270,000 for cytochrome oxidase and succinic dehydrogenase. (See Table IV).

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The last case observed is $\beta$-amylase in barley seeds.[56] Thin slices of seed were bombarded with deuterons, the amylase extracted with papain, and the fraction of activity remaining compared with the activity remaining if the amylase was extracted *before* the irradiation. The results were also compared with data on the inactivation of commercial $\beta$-amylase. In all three cases the amylase was inactivated in the same way, showing that the effect of radiation on the amylase molecule is the same in the seed matrix as in an ordinary preparation.

It must be emphasized that all the experiments discussed were done with the cells or preparations in high vacuum, so that no free water was present. The next obvious extension is to cells in which water is present. In the meanwhile, it can be concluded that in certain special cases the inclusion of enzyme in a biological structure under certain quite restrictive conditions does not affect their radiation sensitivity.

## Future Application of Radiation Studies to Biological Molecules

The list of radiation "molecular weights" given in Table II shows clearly that the technique of dry irradiation gives information which closely parallels information obtained by totally different methods. A worker who knew only the relative sensitive volumes obtained from this kind of radiation data would form a picture of proteins which would agree closely with that available today. In reality, one must concede that all of this work involves the use of hypotheses, which may be more

or less exact, in deducing the size and shape of the protein molecule from the various kinds of measurements. Thus, sedimentation and diffusion data are expressed as sedimentation rates and diffusion constants and these are analysed and interpreted in terms of ellipsoids. X-ray data appear as intensities and angles and can, in principle, be deeply informative as to structure. In practice, the information obtainable is strictly limited by the complexity of the situation, but, nevertheless in one or two cases, useful structural hypotheses are available. In light scattering studies, intensities are transformed to concentrations and hence to particle weights. In all these cases the original data are very indirectly related to the final deduced size and shape. Radiation inactivation falls in this class also. From the nature of the electron, deuteron and alpha particle inactivation curves one can, by an appropriate theory, deduce volumes, sensitivities and thicknesses. Now in all such inferential methods the exactness of our theoretical understanding is most important. The neglect of hydration in sedimentation work can completely change the molecular shape inferred; suggestions regarding phase relations in x-ray scattering may be inadequate and so on. In the same way, radiation sensitivity needs to be understood before confident deductions can be made.

Nevertheless, as the understanding develops, the method will not become worse; rather it may be expected to improve and it is to be hoped that radiation studies will be capable of more precise use in a few years time.

In the meantime, inactivation by radiation deserves a place with other physical methods of study. It has the great advantage that the relation of particle size and shape to *function* is observed. Thus radiation molecular weights which differ from other values (as for insulin) need not be thought of as "wrong." Rather they should be thought of as adding to the understanding of biological action. The great advantages of radiation methods, as applied here, are the absence of need for purified preparations and the ability to use the biological action to separate out the particular unit studied. The disadvantages are in the need for dry, or nearly dry preparations, and for multiple irradiations.

It is felt that interest in this method will quicken as soon as some relation between the structure of a biological molecule and its function is realized. As it stands today, even the long, thin character of nucleic acid is not important in thinking about its action—largely because this is still mysterious. At present it is not known why catalase, urease, trypsin or hemoglobin have their precise sizes and shapes, the only certain knowledge is that they are all proteins. This state of ignorance will not last and will give place to an understanding of the place of size and shape in biochemical action. At this stage, the generality of

the radiation approach, and its convenience, will make a strong appeal.

Some obvious applications of radiation studies involve measurement of sizes and shapes of functioning units actually on organisms. For example, the size of the serological surface units on viruses has been estimated,[57] of the hemagglutinins on some viruses (Newcastle and influenza)[58] and the attachment enzyme sizes for two viruses has also been found.[59] Radiation studies elucidated the nature of the *Kappa* factor in paramecium.[60] Much more of this kind of application is possible.

As soon as the details of some biological consequences of radiation action begin to be understood, quantitative irradiation studies will greatly illuminate the nature of cellular processes. Thus a given dose to a particular cell can be confidently said to have produced certain percentage changes in enzymes, hormones and genes. The particular effect observed, say delayed division or a particular mutation, can then be analysed and its true origin ascertained. There is therefore considerable promise in the radiation approach; enough to warrant considerable care in laying the foundations of knowledge of these processes.

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# STRUCTURE AND FUNCTION OF THE CHLOROPLAST

*J. B. Thomas*

## Introduction

Nearly all the energy needed for life is derived from products of photosynthesis. In nature, light is converted into chemical energy by means of this process in a way unequalled by attempts to do so artificially. The energy conversion takes place within the molecules of the photosynthetic pigment apparatus localized in plastids, small bodies embedded in the cytoplasm of plants. Because of their coloured appearance these structures are called chromoplasts. In higher plants and green algae, they look green and are designated chloroplasts. It is obvious that examination of these organelles is a helpful approach to the study of photosynthesis.

The more techniques are refined, the closer do problems of both structure and function approach each other. This is true of recent investigations on the photosynthetic pigment system, owing to the introduction of the electron microscope, and to progress in spectral research.

This particular situation may render it worthwhile to discuss the researches mentioned in relation to each other. The present review deals with this subject.

Chloroplast studies have been amply reviewed by Weier,[1] Rabinowitch,[2] Frey-Wyssling,[3], [4] Granick,[5] Strugger,[6] Weier and Stocking,[7] and Straus.[8] The reader may be referred to these papers for more detailed discussions concerning earlier investigations. Only as far as they are of interest in relation to the subject in question, will they be mentioned.

## Methods

Due to the small dimensions of the chloroplast structures, their study encounters some methodical problems, which may be discussed briefly here.

*Light microscopy.* The earlier morphological investigations have been done with the light microscope. The results are rather confusing. This is mainly due to the fact that the observability is limited by the resolving power of this microscope. Structures with a diameter smaller than 0·3$\mu$ are inadequately resolved. Actually, the dimensions of the chloroplastic sub-units are of this order of magnitude. The use

of ultraviolet light results in a slight improvement. However, without underestimating the many important investigations done with the light microscope, this instrument is unsuitable for studying the various substructures of the chloroplast.

*Electron microscopy.* With the development of the electron microscope, considerable progress was made. The best instruments allow the observation of particles with dimensions even smaller than 20 Å. However, the new technique was also open to some serious objections. Since this microscope plays an important role in the study of the chloroplast it seems desirable to mention the main deficiencies here.

First, when chloroplasts are to be isolated they are suspended in a liquid medium markedly differing from their natural surroundings. Next, a droplet of this suspension is mounted on the object-bearing film and desiccated. Consequently the tiny structures are subject to considerable surface tensions as well as concentration effects. By using freeze-drying techniques it is possible to prevent the former inconvenience. However, even if the ice formation occurs very quickly, it remains open to question whether the latter one is sufficiently eliminated. Further, the electron beam in the microscope, may markedly disturb sensitive structures. Finally, it should be born in mind that, instead of being photographs, the electron micrographs are mere registrations of the electron scattering power of the preparations.

The above holds for all organic objects. However, this does not necessarily imply that the electron microscope is a useless tool in the study of these structures. Actually, this microscope reveals many details quite reliably. But it should be emphasized that it cannot always be decided with certainty whether the observed structures are real or whether they are artifacts. If so, the use of other methods, e.g. birefringence measurements, is required. If, however, there are no obvious ways of checking the correctness of a conclusion, variations of the preparatory procedure may be helpful. So, with regard to the following discussions, it may be stated here that in many cases the interpretation of electron micrographs includes a more or less hypothetical element.

## Structure

*General appearance.* Anthonie van Leeuwenhoek[9] long ago observed chloroplasts and described them as green globules in plant cells. Although his conceptions were only partly right, this observation yielded two outstanding facts: chloroplasts are definite bodies, and the green pigment is concentrated in them. The latter statement was contested by several older authors, amongst whom Sachs[10] claimed to have observed that the green colour was present throughout the cytoplasm. Nowadays it seems most likely that these investigators

were mislead through observing injured cells. For, in recent years, it was made highly probable,[11–16] that even in blue-green algae and photosynthesizing bacteria, which are truly choroplast-free, chlorophyll is not homogeneously distributed, but is concentrated in units smaller than chloroplasts. It may be remarked that the dark spots in electron micrographs of green bacteria, published by BICKNELL,[12] are considered probably to represent grana by CALVIN and LYNCH.[13] BICKNELL himself is not certain about the nature of the spots. They may represent concentrations of metabolites, e.g. sulphur globules, as well.

Since, in plant cells, the presence of the green colour due to chlorophyll, is always linked to the ability to photosynthesize, this pigment was considered to act as a photocatalyst in photosynthesis. Direct proof of such a relationship was supplied by the coincidence of photosynthesis action spectra and chlorophyll absorption spectra. Papers dealing with action spectra are extensively reviewed by RABINOWITCH.[17] Since in aqueous extracts chlorophyll is closely accompanied by carotenoids,[18–26] it seems right to accept that these accessory pigments occur in the chloroplasts too.

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Chloroplasts may, therefore, generally be described as distinct pigment bearing bodies of primary interest in photosynthesis.

*Shape.* The chloroplasts of different species show a considerable variation of shape; they may look like plates (*Mougeotia*), spiral forming bands (*Spirogyra*), discs or lenses (higher plants), cups (*Chlorella*), networks (*Oedogonium*), stars (*Prasiola*). Size and number per cell may vary to a large extent, too.

*Surface.* The presence or absence of a surrounding membrane has been a controversial matter throughout the study of the chloroplast. MEYEN[27] was the first to mention the occurrence of a chloroplast membrane. NÄGELI[28] confirmed this in a more detailed study. It is a very tiny structure; when the chlorophyll disappears, after the formation of starch grains, in chloroplasts of *Zygnemaceae* and *Desmidiaceae*, it can be clearly observed; the grains are described[29] as being situated in a "thin-walled vesicle." VON MOHL[30] contested the existence of such a membrane. Because adequate reviews exist, we will refrain from citing here the considerable number of earlier papers, dealing with this controversial matter. It may be mentioned that a third viewpoint has also been suggested, viz. that the chloroplasts may be "nude," but they may be surrounded by some cytoplasmic layer differing distinctly from the "common" cytoplasm. Whether this layer belongs to the cytoplasm or to the chloroplast is not clear. The literature in question has been reviewed by MEYER.[31]

Owing to their thickness, it seems doubtful whether chloroplast membranes can be observed under the light microscope. It might be possible only if they were viewed slightly obliquely.

Since, however, observation may be seriously troubled by diffraction patterns, light microscopical study seems to be rather unreliable in this respect. The examination of fixed and stained preparations may also cause misinterpretations. Precipitations may be erroneously considered to represent membranes.

Owing to the preparatory techniques, it may be objected that it is doubtful whether the electron microscope enables a clear decision to be made. Compounds like proteins, lipoproteins and lipids are easily disturbed by the preparative procedure discussed above. Such a disturbance may give rise to the formation of bladders, which might be misinterpreted as membranes. BUNGENBERG DE JONG and WESTERKAMP[32] describe bladders protruding from phosphatide coacervates. Similar bladders could be made to protrude from the surface of *Paramaecium caudatum* by BUNGENBERG DE JONG and HARTKAMP.[33] In their turn, these bladders strikingly resemble those observed by KAUSCHE and RUSKA[34] at the edges of tobacco chloroplasts under the electron microscope. These authors were of opinion that the bladders are lamina, which they supposed to be situated in the interior of intact chloroplasts. MENKE[35] observed these structures in preparations of chloroplastic stroma of *Spinacia* too: ALGERA *et al.*[36] presented some nice electron micrographs of tulip chloroplasts showing these bladders clearly. They observed bladders even larger than the chloroplasts. Actually, this statement proves that these structures cannot represent lamina. According to the authors they probably are phosphatide bladders. Hypotonic medium as well as *p*H are the main factors influencing their formation.

A second type of artifact is encountered in myelin figures, caused by coalescence of lipids. Their contours may strikingly resemble the proteid bladders, but, since they are never folded, they can be recognized as myelin formations. Such "tiny blebs" were seen by GRANICK and PORTER.[37] However, they supposed that these blebs represent small vacuoles in the chloroplast or are formed secondarily as precipitation membranes. The occurrence of myelin figures is clearly demonstrated by FREY-WYSSLING and MÜHLETHALER.[38]

These authors also published an electron micrograph of a folded membrane enclosing the chloroplast content. They considered this structure to represent the true chloroplast membrane. Later, FREY-WYSSLING and STEINMANN[39] abandoned this conception; they assumed all similar formations to be artifacts. However, they did not reject the occurrence of a chloroplast membrane with certainty. The authors pointed to the fact that, in a beautiful electron micrograph of a cross-section of a tulip chloroplast, made by STEINMANN,[40] "condensation" of stroma matter occurs at the surface of the plastid.

THOMAS, BUSTRAAN and PARIS[41] claimed to have shown electron

micrographs of true membranes of spinach chloroplasts. This claim is based on two features. First, the three-dimensional shape of the folds in the upper part of the "bladder" represented in their Fig. 1 seems to indicate that the membrane is a relatively strong structure. This may become more evident if these folds are compared with the "precipitation membranes" as shown in Fig. 16 in the paper of FREY-WYSSLING and STEINMANN.[39] Second, it was shown that this structure consisted of at least two components. Lipoid extraction with benzene did not influence the shape of the membrane, while, after digestion of the proteins by means of pepsin, only collapsed remnants, torn to pieces, were left. The authors therefore concluded that the membrane of the spinach chloroplasts contains both proteins and lipoids. They drew a parallel with the erythrocyte membrane, which, according to WINKLER and BUNGENBERG DE JONG,[42] and to JUNG,[43] consists of a lipoid outer layer and a protein inner one. It may be remarked here that the membrane may be composed of lipoproteins. If so, the molecules will most probably be oriented in such a way that the lipoid groups as well as the proteins are each arranged in a layer.

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For reasons mentioned earlier, some degree of uncertainty remains in the interpretation of electron micrographs. It is therefore of interest that physiological evidence is in favour of the existence of a semi-permeable membrane. KNUDSON[44] stated that swelling of isolated chloroplasts can be prevented in sucrose solutions of suitable concentration. To this phenomenon GRANICK[5] added the fact that seemingly intact chloroplasts can be isolated very easily indeed, free from adhering cytoplasmic strands.

*Discussion and conclusions.* Because of the inadequate resolving power of the light microscope it seems reasonable to attribute no definite value to conclusions obtained with this instrument. Arguments in favour of the existence of a chloroplast membrane seem to be supplied by the dependency of chloroplast size on the osmotic pressure of the medium, by the fact that membrane-like structures, which probably are composed of both proteinaceous and lipoid constituents, are found to occur, as well as by the apparent "condensation" of stroma matter at the surface of the chloroplast. Counter-arguments are: an intact membrane fittingly surrounding a chloroplast has never been observed under the electron microscope, and the preparatory technique required in electron microscopy gives rise to the formation of bladders which may be erroneously considered as membranes.

*Contents.* In former investigations many contradictory statements occur with regard to the question as to whether the contents are homogeneous or whether certain observed sub-units, called grana by MEYER,[45] instead of being artifacts, are essential parts of the chloroplast. The earlier electron microscopical studies suggested that

chloroplasts always contain grana. However, chlorophyll-bearing plastids, in which grana are undoubtedly absent, were recently described. The following deals first with grana-bearing chloroplasts, cf. Fig. 1; next, the grana-free plastids, cf. Fig. 2, will be discussed.

1. *Stroma.* The original definition of "stroma" by MEYER[45] is still most suitable nowadays. It is defined as the colourless substance embedding the green parts of the chloroplast. As to its nature, the literature is rather confusing. This is mainly due to the fact that many observations were made by examining fixed and stained preparations, as well as to the inadequate observability allowed by the light microscope. Since they are amply reviewed, these investigations will not be considered here. However, one type of light microscopical examination, viz. the studies on birefringence, may be mentioned here briefly.

In general, chloroplasts are negatively birefringent.[46–51] An exception is encountered in chromoplasts of brown and red algae which, according to MENKE and KOYDL,[52] show a positive birefringence. MENKE[53] concluded from imbibition experiments with osium-fixed chloroplasts, in which the negative double refraction turned positive, that the original one is a form-birefringence whilst the induced positive double refraction may be due to oriented anisotropic lipid molecules. However, fixation, as well as imbibing, introduces a factor of uncertainty. FREY-WYSSLING and STEINMANN[51] state that the birefringence of *Mougeotia* chloroplasts is mainly a form double refraction, caused by protein layers alternated with poorly oriented lipid ones.

As remarked by FREY-WYSSLING,[4] the birefringence of the chloroplasts indicates that either the complete chloroplasts or some regularly arranged sub-units in them, are uniaxially negative. Certainly, it is not permitted to conclude that the stroma consists of molecular layers. There are, however, some indications of the occurrence of layers. MENKE and KOYDLE[52] as well as MENKE[53] may have observed such lamina. THOMAS, BUSTRAAN and PARIS[41] published an electron micrograph of parts of tiny protein films showing a macromolecular structure, which occur within the stroma of the spinach chloroplast. The authors remarked that it is not possible to conclude anything about orientation of these layers *in vivo*. Possibly they may represent fragments of lamina. Finally, if one examines the above mentioned electron micrograph of a cross-section of a tulip chloroplast, published by STEINMANN[40] and FREY-WYSSLING and STEINMANN,[39] one may notice that the grana seem to be arranged in more or less parallel planes. There may be a slight indication that, in the micrograph, the cross-sections of these planes are represented by lines due to concentration of stroma matter. If this interpretation were true, the grana are suspended in the lamina in a way as suggested by MENKE.[54] However, the electron micrograph in question shows that the stroma may contain

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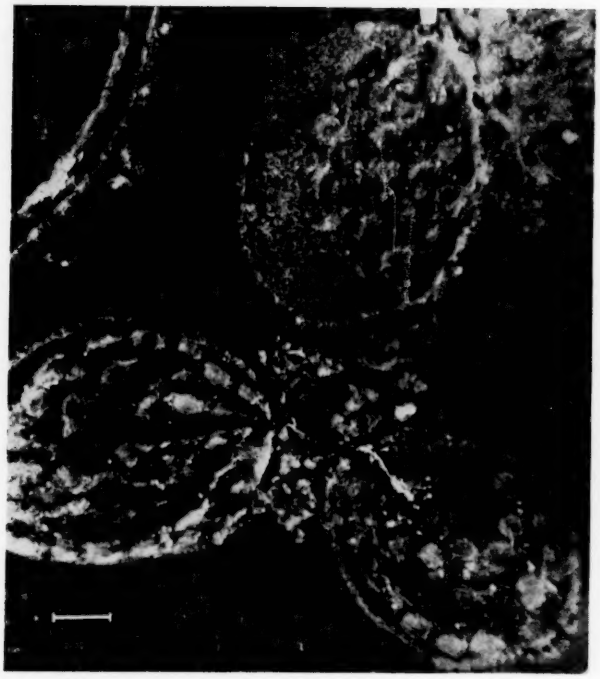

Fig. 1. Cross-section through grana-bearing, chloroplasts, *Spinacia olevacea*

Fig. 2. Cross-section through a lamellate chloroplast, *Euglena spec.*

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a few limina at best, instead of being composed of these structures. Furthermore, this micrograph clearly demonstrates that, contrary to the assumption of MENKE, the grana are definite structures; their occurrence is not due to a heterogenous distribution of certain components within the lamina.

As to the main body of the stroma, very little is known. According to MENKE,[35] who assumed that the chloroplast consists of lamina, the elementary units are rod-shaped particles, presumably protein molecules. In a non-shadowed electron micrograph (his Fig. 9) these "rods" are shown. However, the picture suggests that the shape of the particles seems rod-like because of astigmatism of the electron microscope.

ALGERA *et al.*[36] presented electron micrographs showing heterogeneity of the (dried) stroma in which small globules with a diameter of about 25 m$\mu$ are apparent. Much attention has been paid to the property of the stroma to swell and to "vacuolate" in distilled water and in hypotonic solutions. GRANICK and PORTER[37] demonstrated that the stroma swells more readily than the grana do. However, STEINMANN[40] and FREY-WYSSLING and STEINMANN[39] showed that, in distilled water, the grana also may swell. From the structure of the blebs, STEINMANN[40] assumed that globular particles are responsible for the swelling phenomenon.

In general, it may be mentioned that one should be very careful with regard to conclusions concerning the shape of macromolecules, drawn from electron micrographs. A critical survey of this matter is given by SADRON.[55] If only one of the dimensions of a particle, e.g. a long chain molecule, is considerably larger than the limit of resolution of the electron microscope, whilst the others are too small, the macromolecule will remain invisible. On the other hand, according to SIEGEL, JOHNSON and MARK,[56] a chain molecule may curl up, due to drying, and appear as a spherical particle.

FREY-WYSSLING and MÜHLETHALER[38] concluded that the stroma consists of both proteins and lipoids, or, presumably, lipoproteids in which the linkage between both lipoid and protein molecules is weak. THOMAS, BUSTRAAN and PARIS[41] removed either the proteinaceous or the lipoid components of the stroma by pepsin digestion and lipase digestion, or lipoid extraction with organic solvents, respectively. After removal of the proteins an amorphous sponge-like lipoid mass remained. The "channels" may represent holes remaining after the digestion of proteinaceous strands connecting the grana with each other, cf. also GRANICK and PORTER,[37] and forming a framework (cf. also refs. 39 and 40). It is remarkable that myelin formation was not observed in these preparations. This proteinaceous framework strikingly resembles that shown by LEHMANN and BISS[57] in the



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*Tubifex* egg. The fibrils look heterogeneous; they suggest a similarity with cytoplasmic structures composed of tiny "globules" linked together by "threads." In literature, reviewed by MONNÉ[58] and RONDONI,[59] the "globules," called "chromidia," are said to be autoreproductive and to represent centres for respiration and growth. They are supposed to contain phospholipids, ribonucleic acid, and a large amount of calcium. If so, they must cause a larger electron scattering than the linking fibrils, the "interchromidia," do. BUSTRAAN, GOEDHEER and THOMAS[60] showed that the electron scattering power of the "chromidia" amounts to 1·4 times that of the "interchromidia." Whether this phenomenon is due to a closer packing of the molecules in the "chromidia," or to the occurrence of substances of high electron scattering power, cannot be settled in this way. It might indicate[39] that the stroma proteins are heterogeneous.

However, evidence obtained up till now seems to indicate that the stroma, plays no important rôle, or no rôle at all, in photosynthesis. As soon as chloroplasts are removed from the cells, photosynthesis does not proceed any longer. However, the ability to perform the HILL reaction, the photochemical cleavage of water, presumably representing the primary photochemical step in photosynthesis, is retained. But this process does not depend on the presence of the stroma either. According to ARONOFF,[61] isolated grana, even fragments of them (cf. refs. 62–69) are also able to perform this reaction.

2. *Assimilates and pyrenoids.* Since these bodies are not encountered in all chloroplasts they do not fit into a general scheme of the composition of the chloroplast, hence only a brief mention may suffice here. SACHS[70] was the first to observe starch grains in chloroplasts. ZIRKLE[71, 72] noticed them in chloroplasts from cells of the bundle sheath and of the mesophyll of *Elodea* and maize. According to ZIRKLE a central vacuole, containing a single starch grain, occurs in the *Elodea* chloroplasts. After removal of the starch the vacuole remains visible. Starch grains in *Elodea* mesophyll cells were observed by ROBERTS[73] too. RHOADES and CARVALHO[74] studied the sheath cell plastids in several grasses. They did not notice any starch in the mesophyll plastids. Only when a strong 27 per cent sucrose solution was applied was some starch observed (after 30 hours). The bundle sheath chloroplasts were found to contain up to 40 starch grains. These plastids are weakly green. Apparently they are bodies specialized with regard to the elaboration of starch.

Starch grains are developed from proteinaceous bodies, the pyrenoids. Data concerning algal pyrenoids are reviewed by FRITSCH.[75] In higher plants they were observed by MCALLISTER[76, 77] and MA[78, 79] whilst STEINMANN[80] published an electron micrograph representing a cross-section of a *Spirogyra* pyrenoid.

3. *The grana.* The first description of grana was given by MEYER.[45] Since, in many cases, grana could be made visible by swelling or injuring seemingly homogeneous chloroplasts, later investigators considered them artifacts. This conception lasted until grana were rediscovered by HEITZ,[81–83] DOUTRELIGNE,[84] and WEIER.[85] These observations were confirmed by many others.

According to STRUGGER[6] the grana are suspended in lamina in such a way that they are arranged in piles. This author did not agree with the conception of DE REZENDE-PINTO,[86], [87] who assumed that the grana are suspended in fibrils which are arranged in a helical way. The electron micrograph of a cross-section of a tulip chloroplast, produced by STEINMANN[40] favours the view of STRUGGER, though piles of grana are not observed.

The electron microscope reveals grana as disc-like structures fairly uniform in size within a plant species. MENKE[35] published the first electron micrographs of grana. Because of the occurrence of "lamellae" of the size of grana, MENKE suggested that grana may contain protein lamellae. Whether these lamellae are stroma artifacts or granar lamellae indeed, is difficult to conclude from non-shadowed preparations, the more so, since no acceleration potential is mentioned. ROBERTS[88] believed that grana, termed "plastid granules," are composed of, successively primary, secondary, tertiary, quaternary, quintary, and even senary granules; the latter ones being $0{\cdot}01\mu$ in size. However, judging from the electron micrographs the sub-units are probably stroma particles. GRANICK and PORTER[37] obtained the first electron micrographs of shadowed grana. They reported that, in the vacuum-dried state, the grana of spinach chloroplasts are dense wafer-shaped structures with diameter and thickness of about 600 and 80 m$\mu$ respectively.

In non-shadowed preparations the electron scattering power of the individual grana from any one chloroplast is rather different. Because of the above mentioned dimensional constancy of the grana within each chloroplast, the question arises as to whether the differences of electron scattering properties might be due to different chlorophyll concentrations. This problem still remains to be solved. The density of the grana is considerably diminished by extraction with methanol. Shadowed preparations revealed a clear reduction of the dimensions. Assuming that only fat-soluble components were removed in this way, GRANICK and PORTER concluded that something less than one-half of the granar volume consists of proteins. Their conception that proteins possibly form a framework in which the fat-soluble substances may be arranged, was eventually disproved. The results of ALGERA *et al.*,[36] working with tulip chloroplasts, did not confirm the view of ROBERTS[88] concerning the composition of grana. No sub-units of successively



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smaller dimensions were noticed. The authors did not observe any internal structure in the grana. However, FREY-WYSSLING and MÜHLETHALER[38] showed that grana of tobacco chloroplasts consist of protein lamellae. Moreover, because of the high lipoid concentration as well as for polarization-optical reasons,[3], [51] it was suggested that grana are composed of alternating protein and lipoid layers in which the lipoid molecules may be arranged perpendicularly to the protein lamellae. THOMAS, BUSTRAAN and PARIS[41] confirmed the occurrence of such protein lamellae in grana of spinach chloroplasts. Myelin figures were often found to protrude from grana disintegrated by supersonic treatment. The authors did not succeed in obtaining information on the way in which the lipoids are arranged in the granum. These substances may occur freely, as accepted by FREY-WYSSLING and MÜHLETHALER,[38] or, in combination with the proteins they may form lipoproteins. SJÖSTRAND[89] observed lipoid "knobs" between pairs of protein discs in the outer segments of the retinal rod of the guinea pig eye. Such "knobs" were never observed between the granar lamellae. The conception of the Swiss authors therefore appears quite plausible. After pepsin digestion of spinach grana THOMAS *et al.*[41] noticed lipoid artifacts strikingly resembling those described by FERNÁNDEZ-MORÁN.[90] This author mentioned that, after trypsin digestion of vertebrate nerves, the ordinarily compact lamellae disintegrated into rod-shaped elements, which stained intensely with osmic acid. Such lipoid rods, remaining after pepsin digestion of grana and arranged in a fan-like fashion, might provide some indication that, originally, the lipoid compounds were linked to the protein particles of the lamellae.

In the same way as it was done with the chloroplast membranes, evidence of the occurrence of a granar membrane consisting of proteins and lipoids was procured by THOMAS, BUSTRAAN and PARIS.[41] It was suggested that the lipoids may form the outer layer of the membrane. During the first stage, lipoid extraction or digestion often caused the grana to become loosened from the stroma, leaving cavities behind. This phenomenon may indicate that a delicate lipoid outer layer is removed and, consequently, the linkage between granum and stroma is disturbed. After extraction with alcohol WIELER[91] noted these cavities under the light microscope.

Since the isolated grana escaped visibility WIELER erroneously believed that they were totally dissolved; consequently, he considered the grana to be composed mainly of lipoids.

Working with *Aspidistra* grana FREY-WYSSLING and STEINMANN[39] succeeded in demonstrating that the protein lamellae consist of two macromolecular layers. This is shown by an electron micrograph of a lamella, fixed with osmic acid, in which a fragment of one of these layers was torn off. At this spot the second single layer is clearly

visible. The authors demonstrated the occurrence of two layers per lamella also in quite a different way. Grana were allowed to swell in distilled water. This process was observed under a phase contrast microscope; the grana grew out into long thread-like structures. Electron micrographs revealed that these "threads" consisted of vesicles representing swollen lamellae still more or less attached to each other. Spinach grana showed the same phenomenon though to a lesser degree.

The thickness of lamellae from *Aspidistra*, spinach and algae was determined to be about 70 Å by Steinmann.[40] Consequently the thickness of the single layers amounts to about 35 Å. Frey-Wyssling and Steinmann[39] remarked that the diameter of these layers equals that of a dry protein particle with a sedimentation constant of one Svedberg-unit. Since, in a dry state, the globular macromolecules, which are considered to be the building stones of the lamellae, will be flattened, the authors concluded that, *in vivo*, the lamellar thickness may considerably surpass 100 Å.

*Otherwise-structured plastids containing chlorophyll.* As mentioned above, in studies with the light microscope many chloroplasts appeared to be homogeneous. At first, electron microscopy suggested that in these chloroplasts grana were not observed under the light microscope because of the insufficient resolving power of this instrument.

Recently, however, Steinmann[80] presented electron micrographs of cross-sections of *Mougeotia* and *Spirogyra* "chloroplasts" showing a merely lamellar structure. Neither stroma substance nor grana were observed. In some parts the lamellae were bundled to a more or less degree. These spots show an irregular honey-comb structure. In the middle of the "cells of the honey-comb," tiny "threads" are visible arranged perpendicularly to the lamellae. The author stated that these structures were observed much more frequently in relatively thick cross-sections than in thin ones. It may therefore be justified to suppose that these "honey-combs" are artifacts. Without doubt, the regular orientation of the lamellae is interrupted by pyrenoids. Moreover, the micrograph representing a cross-section of a pyrenoid containing part of a *Spirogyra* "chloroplast" provides evidence that these plastids too, may be surrounded by a membrane.

The thickness of the lamellae was determined to be about 70 Å, the same value obtained for vacuum-dry lamellae from grana. Consequently, *in vivo*, this dimension may also surpass 100 Å. Steinmann pointed to a possible significance of the fact that "chloroplasts" as well as retinal rods, both organelles adapted for converting light into chemical energy, are composed of lamellae. Wolken and Palade[92] cross-sectioned chloroplasts of two flagellates, *Euglena* and *Poteriochromonas*, which also proved to consist of lamellae. The preparations

were non-shadowed, and this fact renders the interpretation of the electron micrographs somewhat more difficult. The authors mentioned "chloroplast granules" occurring between the lamellae. The micrographs, however, suggest that these "granules" are constituents of the protoplasmic structures forming a more or less transparent background. Pyrenoids were also observed. The authors stated that the chloroplasts are surrounded by a membrane. Unfortunately, reproduction of the micrographs caused the loss of too much detail, therefore it is hardly possible to distinguish any membrane in the pictures. The thickness of the lamellae proved to vary between 180 and 320 Å. Since the angle of cutting was not controlled, however, chloroplasts may often have been sectioned on the bias. The authors stated that, consequently, increased thickness may be shown. They therefore considered the smallest values to be the more reliable ones. It is difficult to measure membrane thicknesses in unshadowed preparations. One is never certain to measure the thickness of a single membrane instead of that of a couple of membranes pressed together. Comparison with the electron micrographs published by STEINMANN[80] suggests that the thickness of the lamellae of the chloroplasts in question amounts to about 70 Å also in a dry state.

*Recapitulation and conclusions.* The contents of the chloroplast consist of the stroma in which the grana are embedded. Probably the grana are suspended in membrane-like layers, called lamina. A granum is composed of a pile of disc-shaped protein-layers, the lamellae, most likely surrounded by a granar membrane. Each lamella consists of two protein monolayers. Both stroma and grana contain either proteins and lipoids, or lipoproteids.

Chlorophyll bearing plastids, consisting of densely packed lamellae, are observed in some flagellates and algae. These structures are commonly designated as grana-free, lamellar, or homogeneous chloroplasts.

Both kinds of these organelles have three features in common: they are the bearers of the photosynthetic pigment system; they contain protein lamellae with a thickness of the order of 100 Å, and they are able to perform the photolysis of water. They differ with regard to the arrangement of the lamellae, which are either restricted to the grana or completely fill the organelle in question.

It appears, therefore, that two types of "chloroplasts" are able to perform the same reaction. Since this reaction represents the primary photochemical act in photosynthesis, this statement seems to be somewhat astonishing. One might expect this basic process of absorbing light and converting it to chemical energy to be established by fundamentally equal organelles. There are more reasons for believing so. In order to discuss them it is necessary to consider first some functional features of the chloroplasts.

## Functional Data

*Introductory remark.* The function of the chloroplast consists in enabling photosynthesis to take place and, at least, in establishing the primary photochemical step, the cleavage of water. Since it is beyond the scope of this review to discuss the various aspects of photosynthesis, only those physiological, physical and chemical investigations that may elucidate structural problems will be dealt with here. First, the composition of the pigment apparatus may be discussed.

*Chemical composition of the chloroplast.* According to Mommaerts[93] chloroplasts contain 75 per cent water. Analyses of the dry matter have been carried out by many authors. These studies were amply reviewed (see refs. 2 and 5). One may say that, on a dry weight basis, chloroplasts contain roughly 50 per cent proteins, 30 per cent lipoids and 10 per cent pigments. Three classes of pigments are known to occur in the photosynthetic apparatus. Apart from chlorophylls, accessory pigments (the carotenoids) are always present. Moreover, phycocyanin and phycoerythrin, both phycobilins, are found in red algae, while blue ones contain the former pigment. Literature concerning the nature of the pigments has been amply reviewed (refs. 2, 94–96). Moreover chloroplasts contain some vitamins (refs. 5, 97 and 98) and many enzymes (refs. 2, 5, 7, 99–101).

*State of the pigments.* In the living cell the chlorophylls as well as accessory pigments occur combined with proteins or lipoproteins. For these complexes various names have been suggested: chromophyll,[102] phyllochlorin,[103] chloroplastin,[104] photosynthin,[105] chloroglobin or chromoglobin[2] and chlorophyllin or chromophyllin.[106] Recently, Takashima[107] claimed to have obtained such chlorophyll-lipoprotein complexes in "crystals."

In more than ten plant species the mass ratio of chlorophyll to protein was found to vary between 0·33 and 0·06.[2] Recently Frey-Wyssling[4] calculated that 9 chlorophyll molecules are present per protein particle of a weight of about 17,000. Takashima,[107] however, determined the presence of 2 chlorophyll molecules per protein molecule of a weight of 19,200. When working with spinach chloroplasts Thomas, Blaauw and Duysens[69] calculated that about 100 chlorophyll molecules occurred per particle of a critical size with regard to the ability of performing the Hill reaction, cf. Fig. 3. The diameter of these particles was found to be approximately 120 Å. Since the weight of a spherical particle with such a diameter can be estimated to be about 50 times that of a protein particle as mentioned above, the latter data seem to support the view of Takashima. It may be remarked that Thomas, Blaauw and Duysens measured the diameter of the protein particles from electron micrographs. They assumed that the measured value coincided with the true one. Evidence that this assumption is



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justified is afforded by experiments of PARDEE, SCHACHMAN and STANIER,[15] and KOMEN,[108] who obtained coinciding values for weights of particles from an aqueous extract of purple bacteria by computing

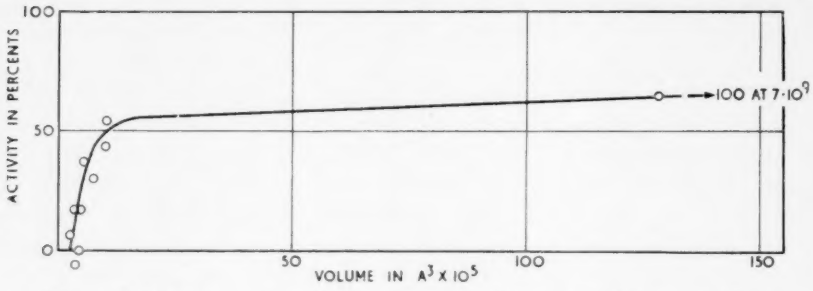


Fig. 3. Relation between rate of oxygen production and particle size. After THOMAS, BLAAUW and DUYSENS.[69]

them from sedimentation velocity as well as by calculating them from measurements of the particle diameter from electron micrographs. These authors found rather high values for the weight of the coloured

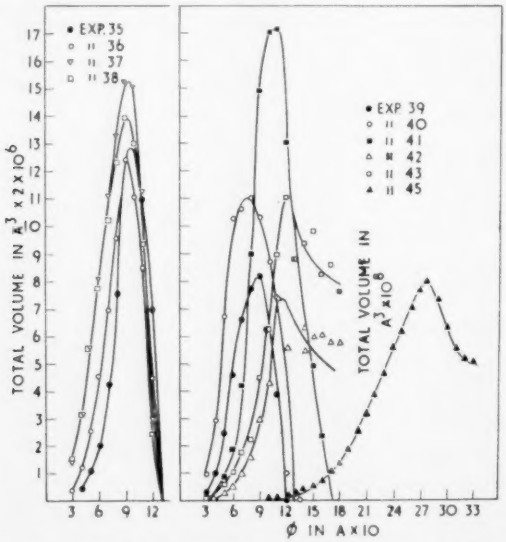


Fig. 4. Various particle sizes in aqueous extracts of spinach leaves prepared by supersonic disintegration and subsequent centrifuging. After THOMAS, BLAAUW and DUYSENS.[69]

particles $3 \cdot 10^7$ approximately. However, judging from the electron micrographs one may ask whether these particles did represent the smallest units instead of grana or grana fragments. As shown in Fig. 4, THOMAS, BLAAUW and DUYSENS[69] demonstrated that suspensions of

particles of various, sharply limited, sizes can be prepared from spinach grana suspensions by supersonic disintegration. It is possible that, in purple bacteria, the size of the protein particles differs from that of higher plants. Evidence for such a difference might be offered by FRENCH[105] who determined a rather low value, of the order of 1:30, for the molecular ratio of bacteriochlorophyll to protein.

Transferring the photosynthetic pigments into a monomolecularly dispersed state by extraction with organic solvents causes a shift of the absorption peaks. This phenomenon is generally ascribed to the fact that, *in vivo*, the pigments are associated with proteins or lipoproteins. ARONOFF[109] and RODRIGO[110], [111] pointed out that, in high concentrations, chlorophyll molecules may associate with each other. The phenomenon mentioned might be explained in such terms too. However, the authors did not succeed in establishing a spectral shift to the *in vivo* position in highly concentrated chlorophyll solutions. These results strongly support the view that the *in vivo* spectrum is a consequence of the complex formation between pigments and proteins or lipoproteins.

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*Transfer of electronic excitation energy in the photosynthetic pigment system.* Chemical energy, needed for the photosynthetic dark processes is obtained from the above-mentioned pigment-protein complexes. Though chlorophylls are the main photosynthetic pigments, the absorption of light, which is active in photosynthesis, is not restricted to them. ENGELMANN[112], [113] already noticed that light, absorbed by accessory pigments, is used in the processes in question. The earlier investigations on this subject were reviewed by MONTFORT,[114] and WASSINK and KERSTEN.[23] However, they are not sufficiently exact to allow more detailed conclusions.

*Carotenoids.* DUTTON and MANNING[115] showed that, in the diatom *Nitzschia closterium*, light absorbed by carotenoids (fucoxanthol mainly) is active in photosynthesis. The photosynthetic efficiencies of light absorbed by fucoxanthol and chlorophyll were thought to be equal. DUTTON, MANNING and DUGGAR[116] measured chlorophyll fluorescence in the same organisms. It was shown that light absorbed by fucoxanthol was as active in inducing chlorophyll fluorescence as light absorbed by chlorophyll itself. This indicates that the excitation energy is transferred from fucoxanthol to chlorophyll with an efficiency of 100 per cent. From these data the authors concluded that light energy absorbed by fucoxanthol exclusively enters the chemical photosynthetic processes via chlorophyll. Independently of DUTTON *et al.*, WASSINK and KERSTEN[23] carried out similar experiments with *Nitzschia dissipata* and *Nitzschia spec.* cf. *ovalis*. From measurements of rates of photosynthesis and fluorescence intensities in various spectral regions, as well as from absorption spectra of cell suspensions, aqueous extracts and pigments dissolved in organic solvents they

concluded that energy transfer from the fucoxanthol-protein complex to chlorophyll occurs. These authors also stated that light quanta absorbed by the fucoxanthol are available for the photosynthesis dark processes only via the intermediary of chlorophyll. In the green-blue and blue parts of the spectrum they found appreciable inactive absorptions. These are due to the presence of other carotenoids that do not, or only to a small degree, transfer light excitation energy to chlorophyll. TANADA[117] discussed the difficulty of determining carotenoid activity in photosynthesis. Overlapping of chlorophyll and carotenoid absorption on the one hand, and shifting of the absorption peaks of the pigments when isolated by extraction with organic solvents on the other, are seriously troublesome in determining the degree of activity of the carotenoids. In particular this holds for determinations made at relatively few spectral regions. Because of this reason TANADA determined the spectral dependency of the photosynthesis quantum yield of the diatom *Navicula minima* var. *atomoides* at a great number of wave lengths (40–50) between 720 and 400 m$\mu$. The quantum yield was found to be constant, about 0·11, from 430 to 680 m$\mu$ except for a "dip" ranging from 520 to 440 m$\mu$ with its largest depth of about 20 per cent, at 475 m$\mu$. Analysing this quantum yield spectrum in combination with an analysis of the absorption spectrum of live cells with the aid of the shifted spectra of chlorophyll *a*, chlorophyll *c*, fucoxanthol, and "other carotenoids" in monomolecularly dispersed state, and assuming an equal photosynthetic efficiency of light absorbed by chlorophyll *a* and chlorophyll *c*, TANADA confirmed the deductions of the aforementioned authors. He concluded that light absorbed by fucoxanthol is as efficient in photosynthesis as light absorbed by chlorophyll. Light absorbed by the "other carotenoids" appeared not to be used in photosynthesis. DUYSENS[118] also confirmed the above conclusions.

Energy transfer from fucoxanthol to chlorophyll is not restricted to diatoms. In the brown alga *Coilodesme californica* HAXO and BLINKS[119] also noticed that "much" of the light absorbed by fucoxanthol is utilized in photosynthesis. Because of the occurrence of only a slightly inactive absorption throughout the region of carotenoid absorption the authors suggested that the "other" carotenoids may be equally active as fucoxanthol. However, since no attempts were made to analyse the carotenoids, the latter conclusion needs confirmation.

The rôle of carotenoids other than fucoxanthol in photosynthesis is less pronounced. EMERSON and LEWIS[120] concluded from quantum yield measurements as well as analysis of the absorption spectra that, in the blue-green alga *Chroöcoccus*, carotenoids are "photosynthetically active" though to a less degree than chlorophyll. Since it is extremely difficult to determine the contribution of the individual carotenoids to

photosynthesis, it may be that some carotenoids are fully "active, while others are not." The authors[121] drew the same conclusion concerning the carotenoids of the green alga *Chlorella pyrenoidosa*. DUYSENS[118] estimated that, in *Chlorella*, the photosynthetic efficiency of carotenoid-absorbed light is about 50 per cent of that absorbed by the chlorophylls. In the green alga *Ulva taeniata* and *Monostroma* HAXO and BLINKS[119] obtained important evidence that light absorbed by the cartenoids is photosynthetically active.

Since in purple bacteria the carotenoid absorption maxima are more pronounced—being more isolated from those of the bacteriochlorophyll—these organisms are quite suitable objects for studies concerning the carotenoid problem. FRENCH[122] who worked with isolated mercury lines, did not observe any participation of carotenoids in the photosynthesis of the purple bacterium *Streptococcus varians*. MANTEN[24], [25] and MILATZ and MANTEN[26] determined the wave length dependency of phototaxis of *Rhodospirillum rubrum* strain 4. Obtaining monochromatic light with the aid of a VAN CITTERT[123] double monochromator and working with the bacteriophotometer they were able to establish an elaborate phototaxis action spectrum. From this spectrum the "activity" of one or more carotenoids became evident. However, the light energy absorbed by the mainly occurring carotenoid, spirilloxanthin, did not enter the photochemical processes. Judging mainly from the facts that bacteriochlorophyll is involved in phototaxis, as well as that the contrast-sensitivity of the bacteria becomes reduced at light intensities at which photosynthesis is saturated, MANTEN suggested that phototactic reactions may be induced by a sudden drop of the photosynthetic rate. Support for this conception was provided by THOMAS and NIJENHUIS[124] and CLAYTON.[125] A photosynthesis action spectrum closely resembling that of phototaxis was established by THOMAS[126] for the same bacteria. The studies mentioned therefore offer an example that, in the same organism, light absorbed by only some of the carotenoids may be active in photosynthesis. According to DUYSENS[118] the effectiveness of light absorbed by the carotenoids is about 40 per cent of that of light absorbed by the bacteriochlorophyll. However, when studying the phototaxis action spectrum of *Rhodospirillum rubrum* strain 1, CLAYTON,[127], [128] noticed an activity of light absorbed by spirilloxanthin in phototaxis. According to this author, strain differences may be responsible for this unexpected result. DUYSENS[118] remarked that the light intensities used by MANTEN were 10–100 times smaller than those applied by CLAYTON and suggested that the contradictory results may be explained by assuming that two bacteriochlorophyll systems, phototactically active under different light conditions, occur in *Rhodospirillum*.



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It may be added that carotenoids in an excited state are probably able to stimulate metabolic processes different from photosynthesis as well. EMERSON and LEWIS[121] observed enhanced respiration in *Chorella* after irradiation with light absorbed by carotenoids. THOMAS and GOEDHEER[129] obtained evidence of the occurrence of such carotenoid activities in the purple bacterium *Rhodospirillum rubrum.*

*Phycobilins.* In red and blue-green algae, the situation is rather complicated, light absorbed by both phycocyanin and phycoerythrin is available for photosynthesis. This was first mentioned by ENGELMANN.[102], [113] EMERSON and LEWIS[130], [120] working with the blue-green alga *Chroöcoccus*, concluded that, within the experimental error, the photosynthetic yield of light absorbed by phycocyanin equals that of light absorbed by chlorophyll.

Photosynthesis action spectra of various red algae were studied extensively by HAXO and BLINKS.[131], [119] It was found that light absorbed by phycoerythrin and phycocyanin is utilized much better in photosynthesis than that absorbed by chlorophyll. The authors remarked that the function of the chlorophyll "is apparently not that of a primary light absorber; this rôle is taken over by the phycobilins." In the red alga *Delesseria occipiens* the rate of photosynthesis, measured as oxygen production, was observed to be equally high in green and red light at saturating incident intensities. According to the authors, this phenomenon may suggest "a common pathway of utilization for light absorbed by different pigments; i.e. phycoerythrin and chlorophyll."

However, fluorescence studies are required to provide more definite information with regard to the way of functioning of the pigment apparatus. VAN NORMAN, FRENCH and MACDOWALL[132] observed that the red fluorescence yield in two red marine algae, *Gigartina radula* and *Iridaea sp.*, is higher when irradiating with green light than with red light. This might indicate that light energy absorbed by phycoerythrin is transferred to chlorophyll. It was shown by DUYSENS[118], [133] that, in *Porphyridium cruentum*, light energy absorbed by phycoerythrin is first transferred, for the main part, to phycocyanin. Next, it is passed to chlorophyll *a*. A similar conclusion can be drawn from FRENCH and YOUNGS' data.[138]

*Chlorophylls.* Energy transfer between chlorophylls, or types of bacteriochlorophyll-protein complexes, was demonstrated by DUYSENS.[133], [118] This author showed that, in *Chlorella*, light of a wave length of 670 m$\mu$, mainly absorbed by chlorophyll *a*, is as effective in exciting fluorescence as light of 650 m$\mu$, which is mainly absorbed by chloroplyll *b*. The fluorescence is due only to chlorophyll *a*. This means that the excitation energy is transferred from chlorophyll *b* to chlorophyll *a* with an efficiency approaching 100 per cent. From the action

spectrum of the photosynthetic yield[121] it was derived that light of both wave lengths is also equally effective in photosynthesis.

In red and blue algae it was found that light absorbed by the phycobilins is often far more efficient in photosynthesis than light absorbed by chlorophyll. This was concluded from the fact that the contribution of chlorophyll to the photosynthesis action spectrum is proportionally less than to the absorption spectrum. FRENCH and YOUNG,[138] and DUYSENS[118] determined some regions of the fluorescence action spectrum of *Porphyridium cruentum*. DUYSENS found that the action spectrum of photosynthesis is similar to that of chlorophyll *a* fluorescence in the red algae *Porphyridium cruentum* and *Porphyra lacineata* as well as in the blue alga *Oscillatoria* sp. The greater contribution of chorophyll *a* to absorption than to the action spectra of fluorescence and photosynthesis indicates that light absorbed by part of the chlorophyll molecules is inactive in fluorescence and photosynthesis. Thus it was concluded that in these organisms, chlorophyll *a* occurs in two different modifications. One of them is both highly fluorescent and highly active in photosynthesis; it receives excitation energy from the phycobilins with an efficiency approaching 100 per cent, cf. also ARNOLD and OPPENHEIMER.[134] The other modification is weakly fluorescent and slightly active in photosynthesis. It is only excited by light absorbed by chlorophyll *a* itself. The energy is "lost." This loss might occur by transfer to an unknown pigment. The light absorption of a pigment that may be chlorophyll *d*, in ether extracts from *Porphyra*, amounted to $10^{-3}$ times that of chlorophyll *a* at the respective absorption maxima. The appreciable fluorescence at 730 m$\mu$ may be due to this pigment. Strong fluorescence of a pigment in small amount can only be due to energy transfer from another pigment, chlorophyll *a*. So in this case there may exist an energy transfer between two chlorophylls, which is "wasteful" with regard to photosynthesis.

According to DUYSENS, the selective energy transfer from the phycobilins to "highly" fluorescent chlorophyll molecules can be explained by assuming that these pigments are located in some part of the pigment structure remote from the weakly fluorescent chloro phyll molecules.

According to WASSINK, KATZ and DORRESTEIN[135] the infrared bacteriochlorophyll absorption spectra of some purple bacteria, for example, the Thiorhodacea *Chromatium* strain *D* and the Athiorhodacea *Rhodospirillum molischianum* show three maxima, whilst only one of them is observed in bacteriochlorophyll solutions in organic solvents. This indicates that the three maxima are due to three bacteriochlorophyll types, each of them showing one infrared absorption peak. The occurrence of the different types may be due to complex formation between bacteriochlorophyll and three distinct proteins or lipoproteins.



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DUYSENS and GOEDHEER[136] demonstrated that the three complexes are equally active in phototaxis, and because of the coincidence of phototaxis and photosynthesis action spectra, also in photosynthesis. DUYSENS[118], [133] studied fluorescence spectra of these organisms and demonstrated that only the bacteriochlorophyll type, called B 890 because of its responsibility for the absorption maximum at 890 m$\mu$, is fluorescent. Light energy absorbed by the other types B 850 and B 800,

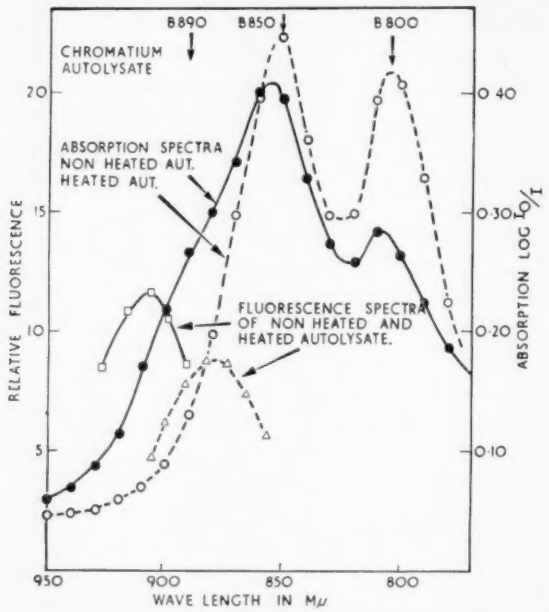


Fig. 5. Evidence of transfer of excitation energy from bacteriochlorophyll type B850 to B890. The absorption spectra show that B890 is destroyed by heating. Concomittantly the absorption maxima of B850 and B800 are increased. The fluorescence optimum of the heated autolysate is shifted to shorter wavelengths, indicating that B850 fluoresces. The fact that in the absence of B890, B850 shows fluorescence suggests that, in the presence of B890, B850 does not fluoresce since it transfers its excitation energy to B890. After DUYSENS.[118]

with maxima at 850 and 800 m$\mu$, is transferred to B 890 with an effectiveness approaching 100 per cent. KATZ and WASSINK[137] found that the mutual height of the infrared maxima can be changed by heat treatment. DUYSENS showed that, by short heating, the B 890 absorption can be made to disappear, whilst that of B 850 and B 800 is increased. The fluorescence maximum of the heated complexes is shifted to shorter wave lengths indicating that, since energy can no longer be transferred to B 890, B 850 becomes fluorescent. This is shown in Fig. 5.

From the parallelism of the degree of chlorophyll *a*—or B 890—excitation and the rate of photosynthesis and fluorescence intensity, DUYSENS concluded that light energy can enter the chemical processes of photosynthesis only via chlorophyll *a*, or in bacteria via B 890. The same conclusion was drawn by FRENCH and YOUNG[138] with regard to chlorophyll *a* in red algae.

*Mechanism of energy transfer.* Different types of transfer mechanisms are discussed by various authors (cf. 106, 118, 134, 138, 139–142). Since it is beyond the scope of this review to enter into the details of this matter, it may suffice to mention here that the observed energy transfer efficiencies can be explained in terms of the theory of inductive resonance.

ARNOLD and OPPENHEIMER[134] applied this idea to energy transfer in the blue-green alga *Chroöcoccus*. FRENCH and YOUNG[138] discussed energy transfer in the red alga *Porphyridium cruentum*. They argued that, because of the good overlapping of both the chlorophyll and the phycoerythrin absorption spectra by that of phycocyanin, transfer by resonance of energy absorbed by phycoerythrin to chlorophyll *a* may proceed via the intermediary of phycocyanin. A quantitative discussion of energy transfer by inductive resonance was given by DUYSENS.[118] In particular, the latter author examined various species of photosynthesizing organisms. He stated that efficiencies of about 100 per cent for transfer of excitation energy from the phycobilins to chlorophyll *a* as well as from chlorophyll *b* to chlorophyll *a* or, in bacteria, from the bacteriochlorophyll types B 800 and B 850 to B 890 render it highly probable that inductive resonance is the only mechanism capable of enabling such highly effective energy transfers under conditions prevailing in photosynthesizing cells.

It may be recalled that DUYSENS also calculated the transfer efficiencies between carotenoids and chlorophylls. These efficiencies were considerably lower than 100 per cent. A discussion of the causes of this phenomenon may be refrained from here.

Because of the poor overlapping of the absorption spectra of the excited pigments with the fluorescence spectrum of chlorophyll *a*, or, in bacteria, of B 890, the energy transfer is unidirectional. Even between the chlorophylls *a* and *b* where the mentioned spectra overlap each other to a higher degree than those of the other pigments and chlorophyll *a*, or B 890, do, the number of back transfers is only very low: DUYSENS[118] calculated it to be $3 \cdot 10^{-3}$ times that of the transfers from *b* to *a*.

Moreover, this author procured experimental evidence that excitation energy may be transferred by means of inductive resonance ultimately to B 890 in bacteria and, by analogy, perhaps not farther than chlorophyll *a* in algae. This means that the energy is not transferred by

this mechanism from these chlorophylls to "reaction centres" involved in the dark reactions of photosynthesis.

*Conclusion.* Calculation[118] showed that, with regard to the chlorophylls, a concentration of the order of 0·1 M/l is required for an efficiency of energy transfer of 100 per cent. Estimations of the chlorophyll content of green cells showed that, according to RABINOWITCH,[2] a concentration of 0·06–0·2 M/l—depending on whether the average chlorophyll concentration in dry chloroplastic matter amounts to 5 or 15 per cent—can be envisaged provided all chlorophyll is located in grana. Recently, RABINOWITCH[66] estimated this concentration somewhat higher: 0·2–0·4 M/l. Since these values also are of the order of the required one, it seems a prerequisite for the functioning of the photosynthetic apparatus that grana are the only bearers of chlorophyll. The studies discussed below demonstrate that this postulate is indeed fulfilled.

*Localization of the pigments.* The light microscope may reveal that the stroma surrounding the grana—provided they are visible—is entirely colourless. This was observed by HEITZ.[81] Since, however, the dimensions of most grana are near to, or below the resolving power of this microscope this statement cannot be made for the majority of chloroplasts. Further evidence is therefore needed. It is provided by chlorophyll fluorescence. Under the fluorescence microscope LLOYD[143] observed that the stroma showed uniform fluorescence. However, with improved equipment, METZNER[144] demonstrated that only the grana are fluorescent. The latter result was confirmed by STRUGGER,[145, 6] DÜVEL and MEVIUS,[146] FREY-WYSSLING and STEINMANN.[39] The latter authors observed fluorescence even in swollen *Aspidistra* grana.

A quite different approach to the problem was made by METZNER.[98] He studied the reduction of silver nitrate to metallic silver by illuminated chloroplasts, the "MOLISCH[147] reaction." Light microscopical examination of the preparations blackened by the metallic silver seems to indicate that the silver is preferently precititated on the surface of the chloroplasts and (or) on the grana in situ. By counting the number of silver precipitations and statistically evaluating these values obtained after irradiation with light of different wavelengths, METZNER established an action spectrum, which is not quite convincing in showing the wave-length dependency of the MOLISCH reaction to coincide with the chlorophyll absorption spectrum. It seems questionable whether this method is sufficiently accurate for the establishing of an action spectrum. However, if one accepts the coincidence of both spectra, and if the interpretation of the light microscopical data is right, the results may indicate that the chlorophyll is concentrated in the grana. The formation of silver precipitates at the surface of the chloroplasts may be explained by assuming that silver nitrate reacts

with a reducing substance, formed during illumination, diffusing—through a membrane—out of the chloroplast. It seems that, in cells, the conditions cannot be sufficiently controlled to obtain or to avoid the formation of surface precipitates at will.

The MOLISCH reaction was used for the localization of the chlorophyll in lamellae by THOMAS, POST and VERTREGT.[148] Firstly, the action spectrum of silver nitrate reduction in suspensions of grana and lamellae was determined by measuring the decrease of light transmission. Lamellae suspensions were prepared from grana ones by supersonic treatment. The action spectrum proved to coincide satisfactorily with the absorption spectrum of such a suspension. Next, preparations of pre-illuminated, silver nitrate-containing, suspensions were studied under the electron microscope. Silver precipitates were observed in single lamellae. Therefore, it seems most likely that chlorophyll is concentrated in the lamellae.

As to the carotenoids no direct evidence with regard to their location was obtained. The action spectrum established by THOMAS, POST and VERTREGT is not sufficiently accurate to allow any conclusions in this respect. However, the aforementioned fact that carotenoids always are found to accompany the chlorophyll-protein complexes in aqueous extracts renders it highly probable that the carotenoids are also bound to the lamellae.

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The phycobilins are extractable much more easily than the other pigments. This might indicate that they occur separately from the chlorophylls. MCCLENDON and BLINKS,[149] however, demonstrated that, in the red algae *Griffithsia pacifica*, *Antithamnion uncinatum* and *Corallina* spec., the pigments were not separated by aqueous extraction provided high molecular weight solutes, such as some polyethylene glycols, dextrin and egg albumin, were added up to a sufficiently high concentration. Thus, this phenomenon indicates that also here the different pigments are localized in the same organelle.

In addition, it may be remarked that the experiments of MCCLENDON and BLINKS seem to support the conception that the plastids are surrounded by a semipermeable membrane.

*Orientation of the pigments.* If chlorophyll molecules are oriented it is expected that they should show dichroism. MENKE[150] noticed a weak dichroism in *Closterium* chloroplasts provided that they contained a relatively low chlorophyll concentration. On the contrary, FREY-WYSSLING and WUHRMANN[151] did not succeed in observing any dichroic effects in algal chloroplasts. For this reason one may conclude that chlorophyll molecules have a random distribution in the plastids.

However, when measuring birefringence, GOEDHEER[152, 153] demonstrated that, *in vivo*, chlorophyll molecules are indeed oriented. He remarked that chlorophyll molecules function as linear oscillators in

the absorption bands, cf. FOERSTER;[154] the electric field, and, consequently, the dielectric coefficient $\varepsilon$, must be different in the oscillation direction and in a direction perpendicular to the former one. According to BECKER[155] resolution of the equation for $\varepsilon$ from the classical theory of electromagnetism:

$$\varepsilon = 1 + C \cdot \frac{1}{\omega_0^2 - \omega^2 + i\gamma\omega} = (n - ik)^2$$

in which $C$ is a constant including the number of active molecules/unit of volume, $\omega_0$ is the resonance frequency, $\omega$ is the frequency of incident light, $\gamma$ is the half width value of the absorption band, $n$ is the refractive index, and $k$ is the extinction coefficient, shows that both $n$ and $k$ are functions of $\omega$, and thus of wave length provided—because of the linearity of the oscillator—the oscillation direction of the linearly polarized incident light has a component coinciding with the oscillation direction of the molecules.

If the mean number of molecules arranged in mutually perpendicular directions is the same—as it is in the case of a random distribution—the refractive indices of the molecules in either direction, are equal; this holds for all wave-lengths. Consequently, the difference of both refractive indices, i.e. the birefringence, is zero for all wave-lengths. If, on the other hand, the molecules are oriented preferentially in one direction, birefringence occurs as a function of wave-length. The same holds for the extinction coefficient. The formula shows that a theoretical relation between dichroism and birefringence occurs. Instead of dichroism one may measure the wave-length dependency of birefringence. With this in mind, it may be remarked that the more difficult dichroism is to measure, e.g. because of high concentration of the pigment molecules arranged in lamellate structures, coinciding with a low degree of orientation, the more preferable is a determination of the wave-length dependence of birefringence.

At a theoretical resonance frequency, a discontinuity in the wave-length dependence of the refractive index and, therefore, of a possible birefringence occurs. The wave-length dependency of birefringence at an absorption band of limited width, the anomalous dispersion, runs as shown in Fig. 6. In this figure the computed values as well as the ones experimentally obtained with chromatographically pure chlorophyll *a*, oriented at ammonia oleate interfaces, are plotted. It may be remarked that corrections as to the non-classical shape of the absorption band were applied.

Fig. 7 represents the anomalous dispersion in *Mougeotia* chloroplasts. The incident light vibrated in the plane of the chloroplasts or perpendicularly to it. After applying a correction for form birefringence (cf. the legend of the figure) GOEDHEER computed both shape and

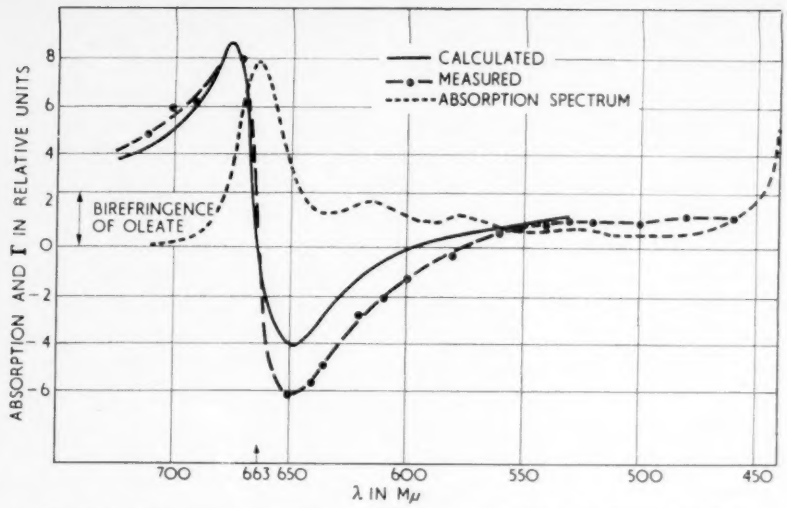


Fig. 6. Anomalous dispersion of birefringence of chlorophyll *a* oriented at ammonia oleate interfaces. The absorption spectrum of chlorophyll *a* in acetone is also shown. The positive birefringence of ammonia oleate is assumed to be approximately independent of wave-length. After GOEDHEER.[152]

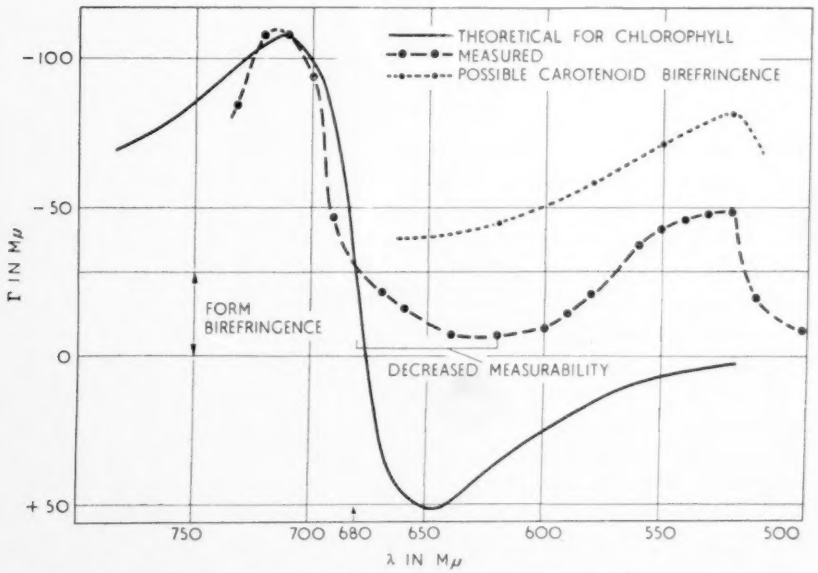


Fig. 7. Anomalous dispersion of birefringence of a *Mougeotia* chloroplast. Because of the unfavourable proportion of high extinction, small Γ, and the amount of depolarized stray light in the spectral region between 680 and 620 mμ, the reliability of the measurements is decreased in this zone. The possible anomalous dispersion of birefringence of the carotenoids was computed from the difference between the theoretical anomalous dispersion of chlorophyll and the experimentally obtained values. After GOEDHEER.[152]

location of the corresponding absorption band. This band proved to coincide satisfactorily with the red absorption peak of chlorophyll. It may be remarked that, according to WIENER'S[156] formula, a possible pseudo-effect of birefringence and dichroism due to lamellate substructures can be estimated to be considerably smaller than the observed anomalous dispersion of birefringence. Consequently, chlorophyll should be oriented in the plastid. However, because of the weak dichroism, if observable at all, the orientation is only partial. As to the mode of orientation, it was concluded that the sum of the components of the "red chlorophyll oscillators" in the plane of the chloroplast surpasses that corresponding with a direction perpendicular to this plane. In an analogous way analysis of Fig. 6 yielded evidence that the carotenoids are also oriented, though to a small degree. The same was found with *Funaria* chloroplasts.

MENKE[150] and FREY-WYSSLING[155] and WUHRMANN[151] observed anomalous dispersion in *Closterium* and *Mougeotia* chloroplasts respectively. However, they did not draw any conclusions from it with regard to the orientation of the chlorophyll.

*Recapitulation.* The main results discussed in this chapter indicate that: (1) the chlorophylls and, most probably, the accessory pigments are concentrated in lamellae, and (2) the chlorophylls and the carotenoids have no random distribution; they are partly oriented.

## DISCUSSION

The aforementioned observations render it highly probable that the photosynthetic pigments are localized in (lipo)protein layers, or at the surface of them. Consequently, it seems right to consider these structures, the lamellae, to be the most fundamental constituents of the photosynthetic apparatus.

The occurrence of the lamellae may be either restricted to certain structures within the chloroplast, the grana, e.g. in spinach, or extended throughout the plastid, e.g. in *Mougeotia*. LEYON[157] assumes that the latter type is primitive. In literature the terms "granulated chloroplast" and "lamellar chloroplast" are used respectively.

However, as remarked by THOMAS, POST and VERTREGT,[148] one may ask whether there is any fundamental difference between a "lamellar chloroplast" and a granum. Both are the only carriers of the photosynthetic pigments in the live cell. Both are built up of (lipo)-protein lamellae. Evidence is obtained that both are surrounded by a membrane. As far as it is obvious at present, the main difference seems to be a matter of size. However, discrimination based on differences in size is rather unsatisfactory. This may be demonstrated in Fig. 8. Here a cross-section of a spinach chloroplast is shown. A granum isolated from such a chloroplast looks like a granum of the "chloroplast-free"

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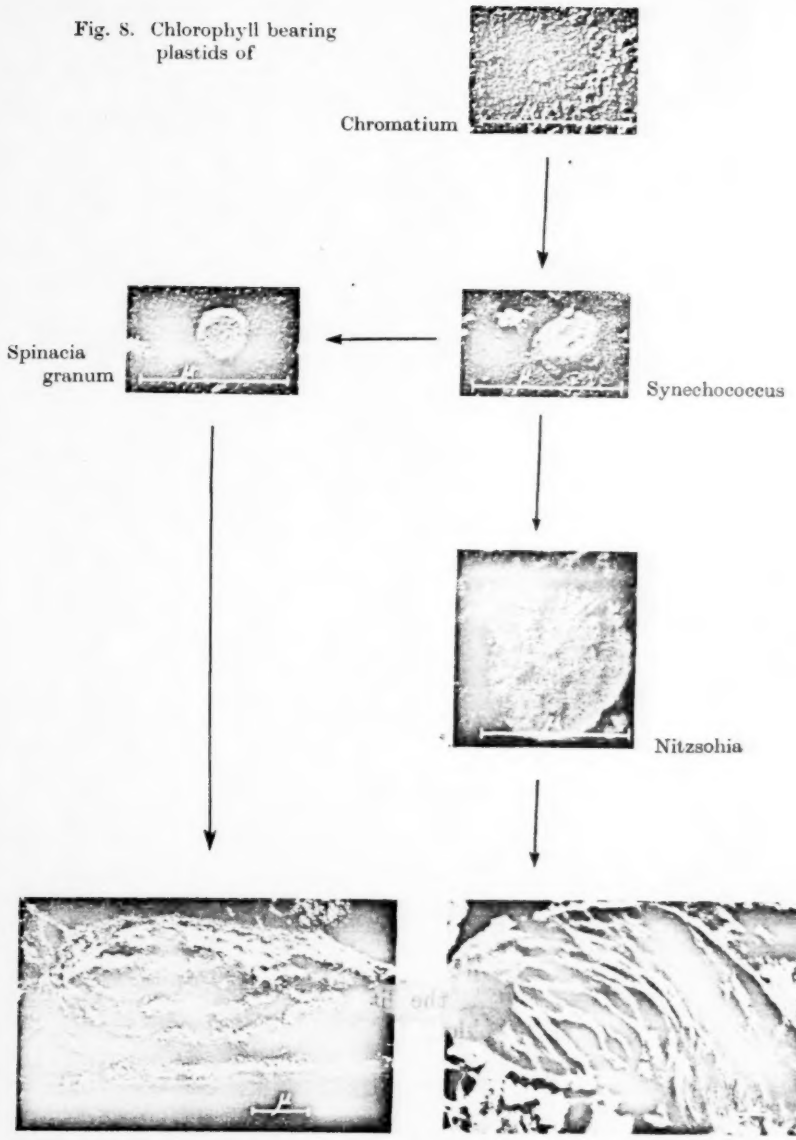
Fig. 8. Chlorophyll bearing plastids of



blue alga *Synechococcus* spec. Free grana may be smaller, e.g. in bacteria, or they may be larger, e.g. in diatoms. Still larger, but essentially the same with regard to structure, are the plastids of *Euglena*. A cross-section of such a plastid is shown too. Due to disturbance by the preparatory technique, the homogeneously lamellate structure is partly split up in bundles. In the literature such plastids are called "lamellar chloroplasts." It is obvious that difficulties may be encountered when using the customary nomenclature. This may be demonstrated most clearly by considering the diatom plastid; with regard to its size it may be designated as "granum" or as "lamellar chloroplast" as well.

The above considerations seem to support the viewpoint of THOMAS, POST and VERTREGT,[148] who suggested that all chlorophyll-bearing lamellar structures be called grana. In this way the term "chloroplast" is restricted to the structures usually called "granular chloroplasts." With respect to the above, chloroplasts may be defined as organelles containing grana embedded in a stroma substance.

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As a consequence of this conception the dimensions of grana may vary within rather wide limits: their volume may fluctuate from about $10^{-3}$ $\mu^3$ in bacteria to $10^3$ $\mu^3$, in *Mougeotia*, or more. This is shown in the right-hand side of Fig. 8. The left-hand side may suggest that a chloroplast does not "fit" in the series of structures in which the primary photosynthetic act occurs. The structures designated by customary terms "granulated chloroplast," cf. *Spinacia*, and "lamellar chloroplast," cf. *Euglena*, look unequivalent.

## GENERAL SUMMARY

Chloroplasts are probably surrounded by a membrane. Plastids, usually called "granular chloroplasts" contain a colourless stroma substance in which grana occur. The grana may be suspended in lamina.

Evidence is obtained that the stroma is heterogeneous. It consists either of proteins and lipoids, or lipoproteins.

The grana are also composed of these constituents. They are built up of a pile of protein lamellae, probably surrounded by a membrane.

Other-shaped chlorophyll-bearing plastids, usually called "lamellar chloroplasts," consist of these lamellae only.

Both kinds of plastids may contain reserve food substances formed in special regions.

From functional data, evidence is obtained that the photosynthetic pigments are concentrated in the grana and, in particular, in the lamellae.

Determination of anomalous dispersion of birefringence indicated that the chlorophylls and, to a minor degree, the carotenoids are partly oriented.

It seems advantageous to discard the terms "granular chloroplast" and "lamellar chloroplast." Instead of them the terms "chloroplast" and "granum" may be used respectively.

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# THE THEORY OF X-RAY FIBRE DIAGRAMS

*A. R. Stokes*

## I. General Treatment of X-ray Diffraction by Fibres

### 1. *Introduction*

The fibres encountered by biologists show few of the characteristic properties found in crystalline substances: nevertheless, they give x-ray diffraction photographs that show evidence of considerable regularity of structure on the molecular scale. These fibre photographs differ from normal diffraction photographs of crystals only in their diffuseness, the sharp spots of a single-crystal photograph being replaced generally by broad streaks or diffuse patches in a fibre photograph. This diffuseness indicates that the structure in a fibre is much less orderly than a crystal structure.

The simplest system that gives a fibre photograph is a metal wire, which, owing to the way in which it is drawn out, has certain properties in common with fibrous materials. Fig. 1 shows an example of this, in which the diffraction spots are only slightly diffuse, although with different treatment of the wire they might have been much more diffuse. Fibre photographs of two typical synthetic long-chain organic compounds are shown in Figs. 2 and 3; here the diffuseness is much more marked, particularly in the second of these. Natural biological fibres give very much more diffuse photographs. Thus Astbury's photographs of keratin (Fig. 4), myosin (Fig. 5), muscle (Fig. 6) and fibrinogen (Fig. 7), to give but a few examples, are very unlike anything obtained from a crystalline material. But some biological materials can be modified so as to give much sharper spots; a good example is deoxypentose nucleic acid (D.N.A.), which can be extracted and drawn out into fibres giving diffraction photographs with a wealth of detail such as that in Fig. 8.

From the last-mentioned type of photograph there is some hope of obtaining evidence as to the molecular structure of the material, but with the more diffuse photographs this is seldom possible, since there is insufficient detail in the photograph to provide a basis for structure determination. But the determination of structure (in the sense of finding the precise arrangement of atoms in the material) is not the only use to which x-ray diffraction photographs may be put. They can be used for the identification of certain structural types in different biological materials. A classic example is Astbury's identification[1]

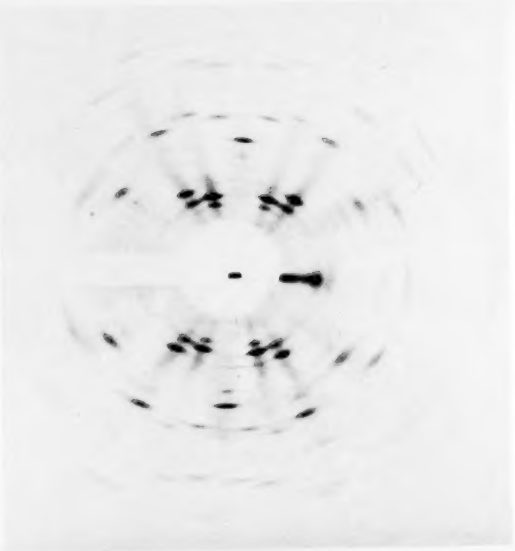

Fig. 1. Stretched aluminium wire.

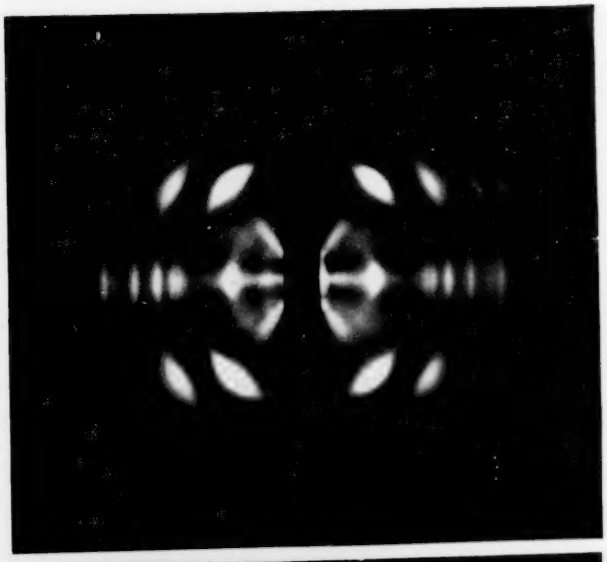

Fig. 2. Poly-vinylidene chloride.

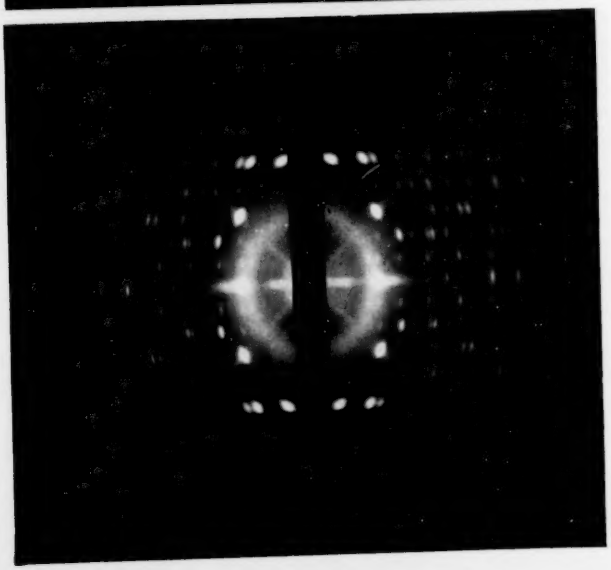

Fig. 3. Polyisobutene (stretched).

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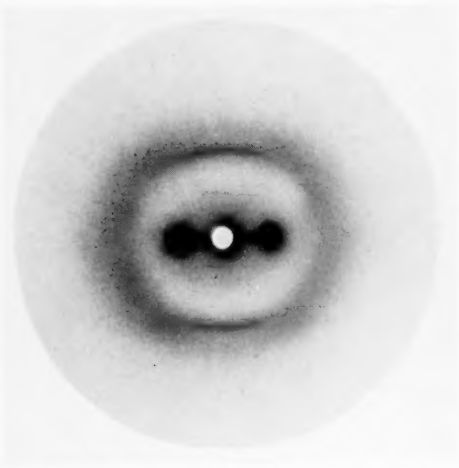

Fig. 4. α-keratin.

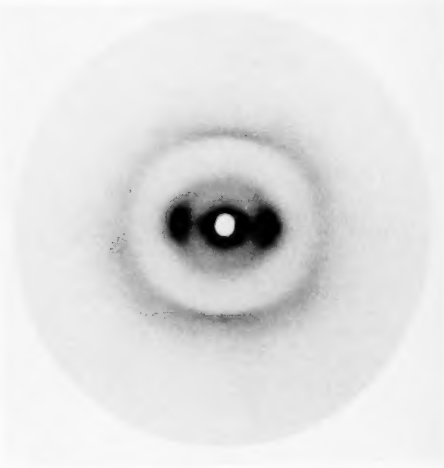

Fig. 5. α-myosin.

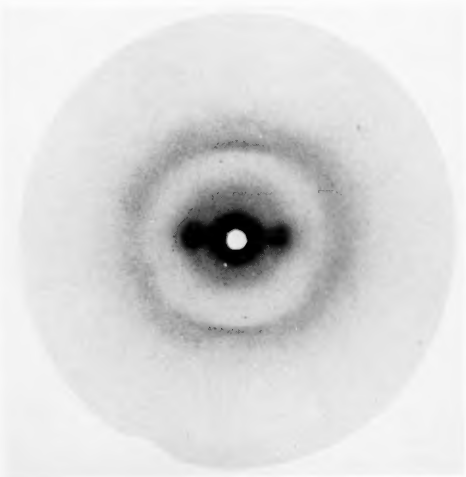

Fig. 6. α-muscle.

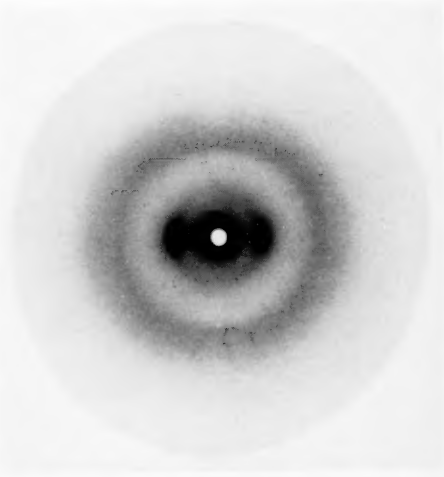

Fig. 7. α-fibrinogen.

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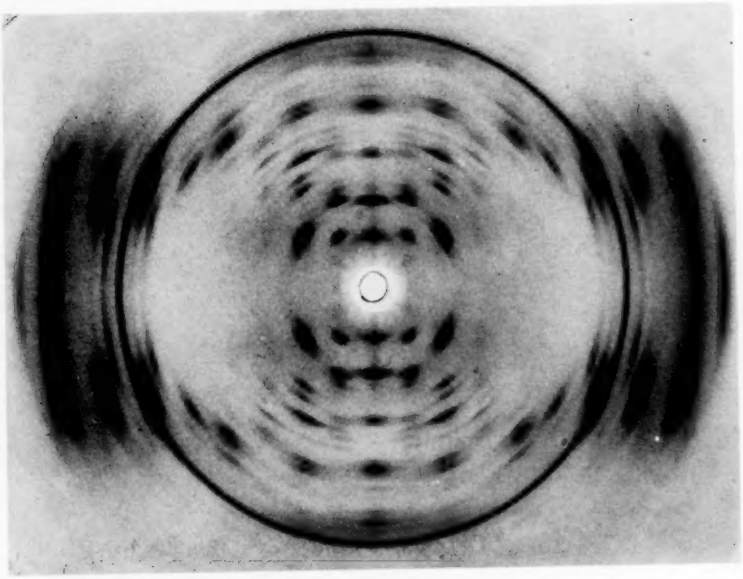

Fig. 8. Diffraction photograph of deoxypentose nucleic acid.

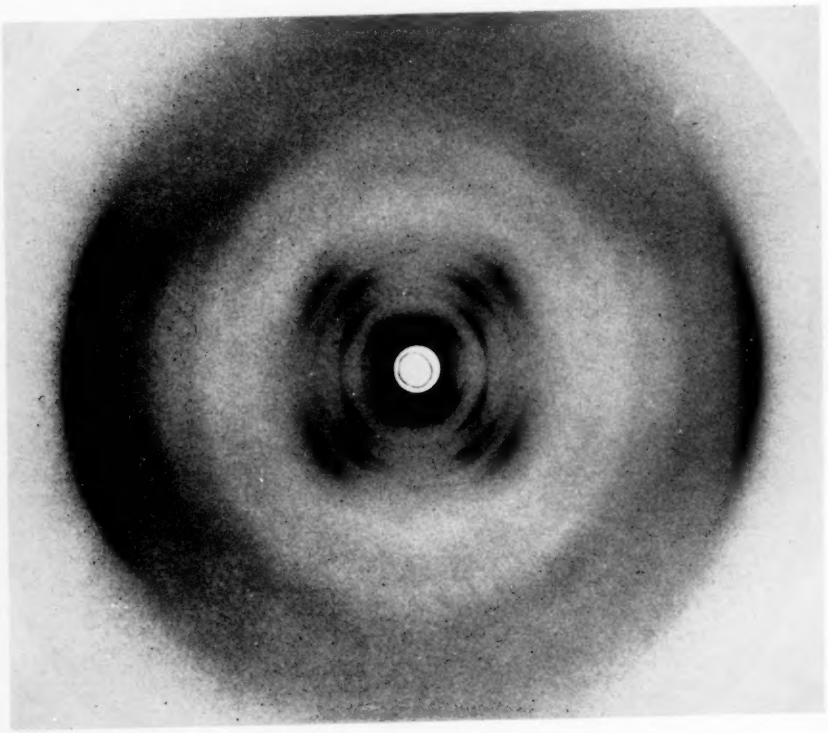

Fig. 9. Sepia sperm head.

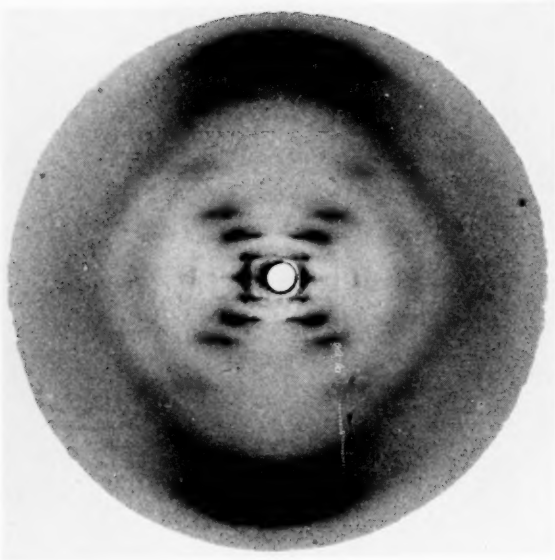

Fig. 10. Wet D.N.A. fibre.

of the keratins, myosins, and fibrinogens as members of a single family. More recently WILKINS and others[23] have shown that the heads of spermatazoa from a large number of different species all give diffraction photographs similar to that of wet D.N.A. fibres obtained by extraction, and this provides striking evidence not only that the D.N.A.'s in all these spermatazoa are similar (though *not* identical) in structure, but also that the structure of the extracted D.N.A. is not much different from that of D.N.A. *in vivo*. Fig. 9 is an example of a spermatazoa photograph, while Fig. 10 shows a photograph of *wet* D.N.A. fibre.

There are also (as we shall see later) certain general features of a structure that can be deduced from a fibre photograph, even when it is clear that the complete structure cannot be obtained.

A fibre is generally assumed to consist of a bundle of *fibrils*, assembled side by side with their long axes parallel or nearly parallel. In each fibril the structure is regularly repeated in three dimensions, as in a crystal, but the fibrils are randomly rotated relative to each other about their long axes. They therefore form a system whose order is intermediate between that of a single crystal, in which the orientation is the same throughout, and a powder of small crystals with completely random orientation.

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An x-ray fibre diagram is generally obtained by setting up a fibre with its axis vertical and directing a narrow horizontal beam of x-rays on to it: the diffraction pattern is then recorded on a flat photographic film behind the fibre or on a cylindrical film surrounding the fibre. The pattern so obtained is the same as the result of superimposing the patterns given by all the fibrils separately, and is therefore identical with the pattern produced by photographing a single fibril while it is rotated about its long axis. It follows that the theory of fibre diagrams is basically the same as that of single-crystal rotation photographs. The essential difference is that, whereas we can stop the rotation of a single crystal and investigate its diffraction in a given position, one is denied the opportunity of doing this with a fibre because of the random orientation of the fibrils.

Many text-books (see, for instance[2–9]) have been written on the subject of x-ray diffraction by crystals and crystal-structure determination, and it is not necessary here to go into details which could be obtained from standard sources. The aim of this article will be rather to give a general idea of the possibilities and the limitations of x-ray diffraction methods, and to deal with the problems peculiar to fibre diagrams.

## 2. *Basic ideas on X-ray diffraction*

X-rays are electromagnetic waves whose wavelengths cover quite a wide range, but we shall be concerned only with those in the wavelength

range about 1–3 Å (1 Å (Ångström) = $10^{-8}$ cm = 0·1 m$\mu$). and in particular the characteristic K$\alpha$ spectral line from an x-ray tube with a copper target, with a wavelength of 1·54 Å, which is convenient to obtain and to use. The distances between neighbouring atoms in a chemical structure are of the same order of magnitude as this wavelength. When x-rays pass through matter, each atom acts as a scattering centre for the waves, and the way in which the scattered waves combine, in any given direction, determines whether or not a diffracted beam will appear in this direction.

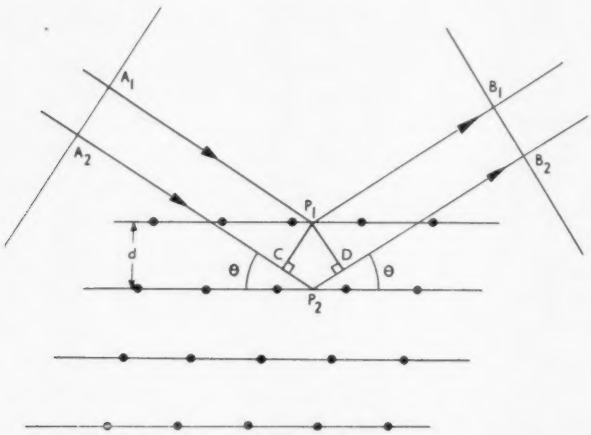


Fig. 11. Reflection of x-rays from a series of parallel planes.

Instead of considering the scattering from individual atoms, it is convenient to consider the waves scattered from a whole plane of atoms at a time. The reason is that a plane of atoms behaves like a partial mirror, reflecting a small part of an incident beam of x-rays according to the ordinary laws of reflection. (It is true that it will act also as a diffraction grating, producing a number of diffracted beams, but this need not concern us here.) A crystal in which there is only one sort of atom, repeated regularly on a three-dimensional lattice, may be divided up into a series of parallel and equally-spaced planes of atoms, as shown in Fig. 11. Let the spacing between the planes be $d$, and let the x-ray beam direction make an angle $\theta$ with the planes. The resultant reflected beam is obtained by combining the waves reflected by the layers. If the reflected waves from all the layers are in phase (that is, if the crests of waves from one layer coincide with the crests of waves from the next) there will be a reflected beam; but if there is even a small phase difference between the waves reflected from one layer and the waves reflected from the next, then the waves reflected from successive planes will be more and more out of phase; the waves will

cancel each other out, and there will be no reflected beam. In order that the reflected waves should be in phase, it is necessary that the path length $A_2P_2B_2$ of a wave reflected from one plane should exceed the path length $A_1P_1B_1$ of a wave reflected from the next plane by a whole number of wavelengths, that is

$$CP_2 + P_2D = n\lambda \quad \text{where } n \text{ is the integer.}$$

or

$$2d \,.\, \sin\theta = n\lambda \qquad \ldots.(1)$$

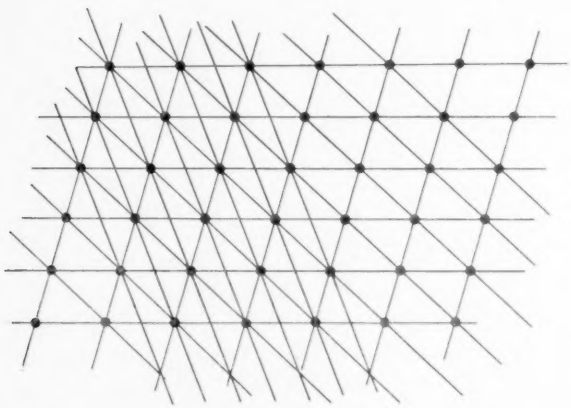

Fig. 12. Parallel planes drawn through atoms in a crystal.

This is known as BRAGG'S LAW.[3] It shows that X-rays can only be reflected from a set of parallel reflecting planes if the glancing angle $\theta$ of the rays on these planes satisfies the condition given by equation (1). If the X-rays are incident at any other angle, no reflection from these planes will appear.

For any regular arrangement of atoms, periodic in three dimensions, there are many different ways of drawing sets of parallel planes through the atoms: some of these are illustrated in Fig. 12. Each set of planes has its own value of $d$, and to each set of planes there corresponds one value of $\theta$ for each value of $n$ in equation (1). A set of planes and a value of $n$ define what is known as an *order of reflection*, analogous to an order of diffraction for a line diffraction grating. Since $\sin\theta \leqslant 1$, it follows from equation (1) that only sets of planes with $d \geqslant \lambda/2$ can reflect X-rays. We can only choose sets of parallel planes satisfying this condition in a limited number of ways, so there is a limited number of possible ways in which X-rays can be reflected.

**3.** Suppose now that the repeated unit of the structure consists of several atoms, which we shall denote by $A$, $B$, $C$, . . ., instead of only one. We can then draw a set of parallel planes of spacing $d$ through all



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the $A$ atoms, as in Fig. 13; a set of parallel planes through the $B$ atoms, another set through the $C$ atoms, and so on, each set with the same spacing $d$. The reflected beam will now be the result of superimposing the reflections from the $A$ planes with those from the $B$ planes, the $C$ planes, etc. It follows that there will be no reflection unless the Bragg equation (1) is satisfied. The angle of diffraction depends only on the repeat distance $d$ and not on the arrangement of atoms $ABC$ . . . in the repeated pattern. The arrangement of the atoms determines the *intensities* of the diffracted beams, since the intensities are determined by the phase differences between the beams reflected from the

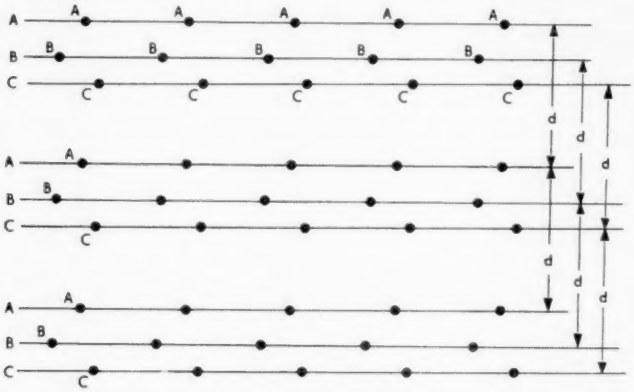


Fig. 13. The reflecting planes in a structure with several atoms per repeated unit.

various planes $A, B, C$. . . . The question of the relation of intensities and structure will be considered later.

4. Thus the appearance of a diffracted beam of X-rays indicates that the atoms of the structure can be arranged on sets of parallel planes whose spacing $d$ is given by equation (1), or

$$d = \frac{n\lambda}{2} \operatorname{cosec} \theta \qquad \ldots.(2)$$

The direction of these planes is given by the fact that their normal bisects the angle between the directions of the incident X-ray beam and the reflected beam reversed, as in Fig. 11.

The study of crystals by X-ray diffraction is concerned essentially with showing up *periodicities* of structure. Distances between two atoms in the structure, such as $A$ and $B$ in Fig. 13, do *not* appear as values of $d$ in the diffraction photograph. The term "spacing" is widely used for the values of $d$ calculated from spots on photographs, but it can be misleading, and "repeat distance" or "periodicity" would be a better one.

5. *Information obtained from the diffraction photograph*

If a single crystal is set up in the path of a beam of monochromatic x-rays (x-rays of one wavelength only) and is rotated, the different sets of planes will come round in turn into positions in which they can reflect the incident x-rays. The reflected beams can be recorded as spots on a cylindrical film placed round the specimen. The position of a spot gives the direction of the reflected beam, and hence we can find the periodicity of the corresponding planes in the crystal, and their orientation *at the time when the planes were reflecting*. But we do not know at what epoch of the rotation the planes were able to reflect the beam.

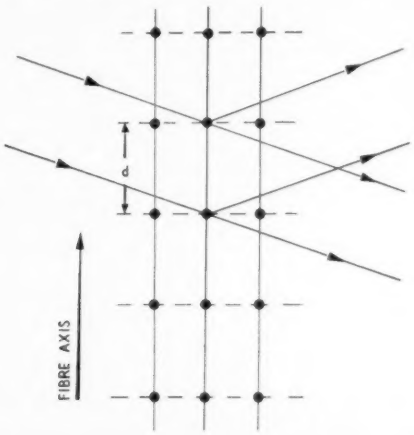


Fig. 14. Reflection from planes perpendicular to the fibre axis.

It follows that we can find the inclination of the planes to the vertical axis about which the crystal is rotated, but we cannot find anything more about their orientation.

When a single crystal is used, there are other methods (e.g. the Weissenberg photograph[2, 8]) of finding the epoch at which the reflections are produced. With a fibre there are no such experimental methods of determining the epoch, since the fibre contains fibrils in all orientations around the fibre axis, and there is no means of knowing which fibril, has produced a given reflection. The missing information can be supplied, in favourable cases, by geometrical arguments, as we shall see later.

However, there are certain features of the structure which can be deduced readily and unambiguously from the fibre photograph.

6. *The longitudinal repeat-distance*

If the fibrils consist of long-chain molecules packed side-by-side as in Fig. 14, we can draw parallel planes perpendicular to the axis of the



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fibre. Let these have a repeat-distance $d$; then X-rays can be reflected by them when the incident and reflected beams both make an angle $\theta$, satisfying equation (1), with the planes. If all the fibrils are accurately parallel and the X-ray beam direction is perpendicular to the fibre axis, this sort of reflection cannot take place because no X-rays fall on the reflecting planes at the angle $\theta$. It frequently happens, however, that the fibrils have their axes disoriented so that some of them are tilted over sufficiently to allow the X-ray beam to be reflected. The

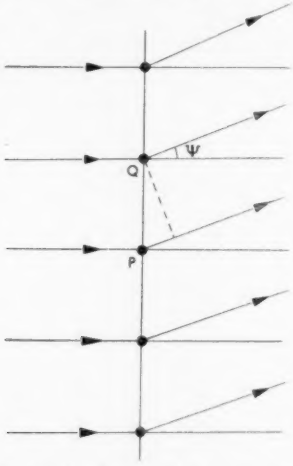


Fig. 15. Scattering from a single row of atoms.

reflected X-ray beams make an angle $2\theta$ with the direction of the incident beam, and the reflection appears on the photograph as an arc with its centre on the meridian (the vertical line through the centre of the photograph).

If the fibrils are not sufficiently disoriented, the meridional reflection can be brought into view by tilting the fibre out of the vertical. If the fibre is tilted through an angle $\theta$ in the vertical plane containing the beam direction, many of the fibrils will then be correctly oriented to give the required reflection. An example of this has been given by PERUTZ.[10]

### 7. *Layer lines*

A single-crystal photograph, taken with one of the crystal axes vertical, has all its spots lying on horizontal lines, of which the equator is one. A fibre photograph will show a similar appearance if the long-chain molecules in each fibril run parallel to the fibril axis, as is usually the case, and if the fibril axes are all parallel. The reason for this can be seen if we consider diffraction from a single vertical chain, as shown in Fig. 15. The waves scattered in any direction making an angle $\psi$ with

the horizontal can only reinforce each other if those scattered from an atom $P$ lag behind those scattered from the next atom $Q$ by an integral number of wavelengths, that is, if

$$c \,.\, \sin \psi = l\lambda \qquad \ldots.(3)$$

where $c$ is the repeat-distance along the chain and $l$ is any integer. If this is a necessary condition for diffraction from one chain, it is a necessary condition for any structure that can be made up of vertical chains of atoms. Thus all the reflected beams must lie on cones of half-angles $90^\circ - \psi$, the values of $\psi$ being given by putting integral values of $l$ in equation (3). These cones intersect the cylindrical film in horizontal circles, which appear as straight lines when the film is opened: hence the appearance of layer lines.

The angles $\psi$ can be obtained from the photograph by measuring the heights of the layer lines above and below the equator. Hence a value of $c$ can be calculated from equation (3).

This is easily done with a single-crystal rotation photograph with sharp spots, but fibre photographs often have their spots blurred by disorientation and other causes, and it may be difficult to pick out layer lines in some extreme cases.

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It should be noted that the meridional reflection may lie slightly off the corresponding layer line. This is because the meridional reflection is due, as explained above, to reflection from a few fibrils with particular orientations, and its angle of elevation above the horizontal is given by $2\theta$, where $\theta$ satisfies the Bragg equation (1). We can see from equations (1) and (3) that $2\theta$ is not quite equal to $\psi$ (for $n = l$); in fact that $2\theta$ must be less than $\psi$. This effect is only noticeable when $\theta$ is not small.

8. *Lateral periodicities, or "side-spacings"*

If a fibril consists of parallel long-chain molecules packed together in a regular arrangement, we can draw sets of parallel vertical planes through them, as shown in Fig. 16, which represents a cross-section of a fibril with two sets of planes shown. These sets of vertical planes will reflect X-rays into horizontal directions, and will thus give spots on the equator of the photograph. The equatorial reflections thus show how the chains are packed together in the fibril. Note that none of the $d$-values deduced from the equatorial spacings is, in general, the distance between chains. Methods of deducing the mode of packing of the chains from the equatorial reflections are given by BUNN (Chapter VI of reference (2) ).

9. *The effect of disorientation*

If the fibrils are not all parallel the spots will be spread out into streaks. If we confine our attention for the moment to one particular set of

reflecting planes of periodicity $d$, we can see that all fibrils with these planes correctly oriented to give a reflection must deviate the X-rays through the same angle $2\theta$, where $\theta$ is given by equation (1). The reflected beams must therefore all lie on the surface of a cone of half-angle $2\theta$ with the incident beam direction as axis. Each spot will be

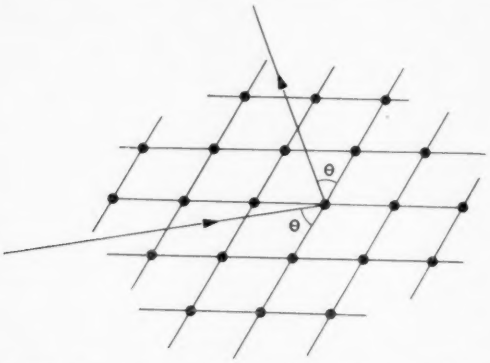


Fig. 16. Cross-section of the vertical diffracting planes.

spread into a streak which is part of the curve in which the corresponding cone cuts the cylindrical film: nearly an arc of a circle, in fact. The relation between the length of the arc and the degree of disorientation of the specimen can be worked out geometrically; it is not a very simple relation, except for the equatorial spots and those near the equator,

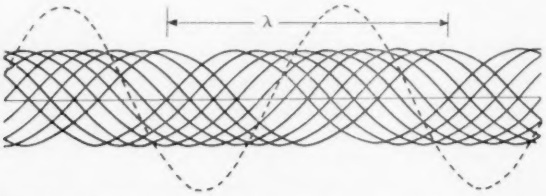


Fig. 17. The resultant of a number of waves with not more than one wave-length phase difference.

where the angle subtended by the arc at the centre of the photograph is approximately equal to the range of angular disorientation of the fibrils. This gives us a method of estimating the degree of disorientation of the fibrils.

10. *Small number of reflecting planes.*

It was assumed in section 2 that the X-rays are reflected from a very large number of planes, so that a small deviation from the Bragg angle of reflection means that the reflected waves cancel each other out and there is no reflected beam. A very small deviation can be tolerated,

however, and this deviation is larger, the smaller the number of reflecting planes in the crystal or fibril. If there are $N$ planes, and if the last of the $N$ reflected waves does not lag behind the first by more than one wavelength, then the $N$ waves will add up to some appreciable amplitude, as shown in Fig. 17.

For reflection at the Bragg angle $\theta$, the last wave lags behind the first by $2(N-1)d \,.\, \sin\theta$, or $(N-1)n\lambda$, and as this is a whole number of wavelengths the waves are in phase. If we increase the glancing angle of incidence to $\theta + \delta\theta$, the last wave will now lag behind the first by

$$\begin{aligned} &2(N-1)d \,.\, \sin(\theta+\delta\theta) \\ &\quad = 2(N-1)d\,(\sin\theta + \cos\theta \,.\, \delta\theta) \\ &\quad = (N-1)n\lambda + 2(N-1)d \,.\, \cos\theta \,.\, \delta\theta \end{aligned}$$

Leaving out the first term, which is a whole number of wavelengths and therefore does not affect the phase relation between the waves, we see that the second term must not exceed one wavelength if the reflected waves are not to cancel out. Since $(N-1)d$ is the thickness $t$ of the set of planes, the condition becomes

$$2t \,.\, \cos\theta \,.\, \delta\theta < \lambda \qquad \ldots.(4)$$

The range of the angle $\theta$ over which appreciable reflection can take place is therefore twice the maximum value of $\delta\theta$, given by

$$2\delta\theta = \frac{\lambda}{t \,.\, \cos\theta} \qquad \ldots.(5)$$

Remembering that the deviation of the X-rays on reflection is $2\theta$, we see that the angular breadth of the reflection is twice $2\delta\theta$, or

$$\beta = \frac{2\lambda}{t \,.\, \cos\theta} \qquad \ldots.(6)$$

In practice it is necessary to adopt some arbitrary definition of breadth for purposes of measurement, and the breadth depends on the crystal shape as well as the size, so we can only take equation (6) as representing an approximate result. More exact treatments have been given by a number of authors (LAUE,[11] JONES,[12] WALLER,[13] STOKES and WILSON[14]), and these show that the numerical constant "2" in equation (6) should be replaced by a number which is generally nearer to unity. There is a lower limit to the broadening which can be detected in the ordinary fibre photograph, and in a typical case the crystals would have to be less than 300 Å thick in order to produce visible broadening. The crystal sizes that can be estimated from the broadening of X-ray reflections are thus always in the far submicroscopical region.



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For a long thin crystal the spots are broadened in a direction perpendicular to the long axis of the crystal. Thus in the case of narrow fibrils the spots are broadened in a horizontal direction. This broadening will be masked in the meridional reflections by the effect of disorientation, but will show up on the equatorial and near-equatorial reflections.

But broadening of the spots is not necessarily a sign of small crystal or fibril size: it may indicate small variations in the periodicities from one fibril to another, giving rise to small variations in the angle $\theta$; or it may be due to distortion or some other form of lack of perfect periodicity within each fibril. In the latter case the quantity $t$ in equation (6) represents the order of the average distance over which the exact periodicity begins to fail. For broadening due to small fibril size, the breadths of the spots are fairly constant so long as $\theta$ is not large (the breadth increases as sec $\theta$ on the equator); for broadening due to slight variations of periodicity or to some forms of distortion, the breadth increases regularly with $\theta$ (it is proportional to tan $\theta$ on the equator). But if "mistakes" are made in the assembly of the structure so as to break up the exact periodicity, it is possible that some spots are broadened while others are not. This subject has been fully treated by WILSON.[9]

It is important to note that the broadening due to the effects mentioned above is always mixed with some broadening due to instrumental factors such as the finite thickness of the specimen and the finite width of the X-ray beam collimator. This "instrumental broadening" must be reduced as far as possible if measurements of the broadening of spots are to be made, but this reduction can only be made at the expense of a reduction in intensity of the diffracted X-rays and therefore means an increase in exposure time. A compromise must be struck between instrumental broadening and length of exposure. Methods of correcting the observed breadths for instrumental broadening have been devised by JONES,[12] SHULL,[15] STOKES,[16] and others.

11. *Upper and lower limits to observable periodicities*

It has been pointed out in section 2 that no reflection can be obtained from sets of planes with a repeat-distance less than $\lambda/2$. This fact imposes a limit to the resolving power of X-ray diffraction, similar to the limit of resolution of a light microscope. In crystal-structure analysis this limit is generally more or less immaterial because the closest distance of approach of any two atoms is always greater than half the wavelengths of copper $K_\alpha$ radiation.

With most natural fibres we find, however, that a limit of resolution is set by the fibre itself, since the intensity of reflections falls off very rapidly with increasing angle $\theta$, at values of $\theta$ around 15° to 20°,

corresponding to periodicities of about 3 Å to 2·3 Å. This sudden falling-off of intensity, which is due to a variety of factors inherent in the fibre structure, makes it impossible to gain any information about periodicities less than those quoted, and means that we cannot hope to "see" individual atoms, but only groups of atoms. A good deal of chemical evidence on molecular configuration must be used in addition to x-ray evidence if we are to fix the positions of all the atoms.

There is also an upper limit to the periodicities we can observe, fixed by the smallest observable deviation of the x-ray beam. The direct beam must be stopped by a beam trap ($T$, in Fig. 18) and a diffracted

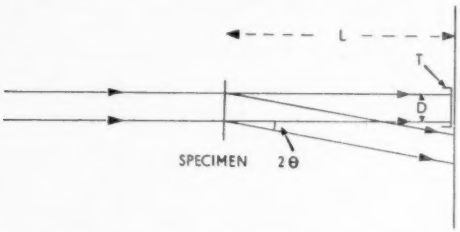


Fig. 18. The minimum observable deviation of a diffracted beam.

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beam, to be clearly recorded, must strike the film clear of this trap. That is, if the beam has width $D$ and the specimen-to-film distance is $L$, the angle of deviation, $2\theta$, of the diffracted beam must be somewhat greater than $D/L$. This lower limit to the angle $\theta$ fixes an upper limit to the periodicity, $d_{max}$, given by

$$2d_{max} \sin \theta = \lambda,$$

or, since $\theta$ is small,

$$d_{max} = \lambda L/D \qquad \ldots.(7)$$

Special cameras have been designed[17] with a long specimen-to-film distance and a small beam width, to record periodicities up to 1000 Å or even 2000 Å. But general-purpose cameras have a much smaller value of $d_{max}$ than this, because the exposure time increases with increasing $L$ and with decreasing $D$, and it is therefore advantageous to make $L/D$ no larger than is necessary.

To sum up: x-ray diffraction cannot reveal detail on a scale smaller than half the x-ray wavelength, nor can it give us any evidence about organization of material on a scale greater than about 1000 A.

## II. The Systematic Determination of Structure from Fibre Diagrams

12. In the following treatment, even more than in the first part of this article, the emphasis must be on general principles rather than on details of structure determination. The detailed methods are fully set

out in several text-books (e.g. [2], [4], [5], [8]) and no useful purpose would be served by reproducing them here. It must be emphasized that crystal structure determination is a specialized subject; it is not merely a technique which can be acquired in a short time. A little knowledge of the methods will not lead to a little success in structure determination: it is more likely to lead to entirely wrong structures.

13. *Determination of the unit cell*

This is the first stage in the determination of any regularly repeating structure. Such a structure may be regarded as being built up of a large number of identical "bricks" packed together; each brick is a parallele-

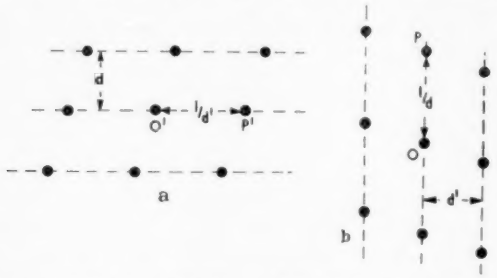


Fig. 19. (*a*) the crystal lattice and (*b*) the reciprocal lattice.

piped and all bricks contain exactly the same arrangement of atoms. Any three edges of the parallelepiped that meet at one corner are called the *axes* of the unit cell, and when their lengths and directions are specified, the mode of repetition of the whole structure is determined.

We have seen in sections 3 and 4 that the directions of the diffracted beams depend solely on the way in which the structure repeats; that is, on the unit cell dimensions. These dimensions are therefore obtained by finding the positions of spots on the diffraction photograph.

We have seen in section 4 that if the directions of the incident and reflected x-rays are known, the periodicity $d$ and the direction of the normal of the reflecting planes can be worked out. Thus we can calculate the periodicities and orientations of all sets of reflecting planes from the photograph. These must be expressible in terms of a unit cell. The unit cell dimensions are most conveniently derived by means of the concept known as the *reciprocal lattice.*

14. The reciprocal lattice consists of a set of points plotted in space as follows. Consider one set of reflecting planes in the crystal, and, starting from an origin $O$, draw a line of length $1/d$,* in a direction perpendicular to the reflecting planes, ending at $P$. If all sets of reflecting planes are considered in turn, and the same construction is carried

* Note that some authors use $\lambda/d$ instead of $1/d$ in defining the reciprocal lattice, but as $\lambda$ is constant this only means a change of scale.

out for each, the result will be an array of points $P$, as shown in Fig. 19 ($b$), and it can be proved that these points are arranged on a regular lattice, the reciprocal lattice. Further, the relation between crystal lattice and reciprocal lattice is a dual one; that is, any point on the crystal lattice can be constructed by drawing from a given origin $O'$ a line $O'P'$, whose length is $1/d'$ and whose direction is perpendicular to a set of lattice planes of periodicity $d'$ in the reciprocal lattice. Thus

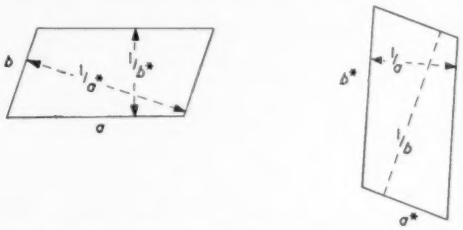


Fig. 20. The crystal unit cell and the reciprocal-lattice unit cell.

the unit cell of the crystal lattice and the unit cell of the reciprocal lattice are related by the fact that the edges of one are perpendicular to the faces of the other, and the lengths of the edges are the reciprocals of the perpendicular distances between the faces, as shown two-dimensionally in Fig. 20.

The construction of the reciprocal lattice from a single-crystal rotation photograph is facilitated by a method described by BERNAL,[18]

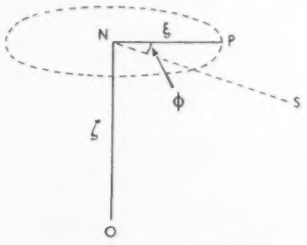


Fig. 21. The coordinates $\xi$, $\zeta$, $\phi$ of a point $P$ on the reciprocal lattice.

in which two of the three coordinates of a reciprocal-lattice point are read off directly from a chart placed over the photograph. These two coordinates are the perpendicular distance $\xi$ of the point from a vertical axis through the origin $O$ (Fig. 21), and the height $\zeta$ of the point above a horizontal plane through the point $O$. The third coordinate may be taken as the angle $\phi$ between the line $PN$ and some arbitrarily-defined direction $NS$ in the horizontal plane through $N$. If $\xi$, $\zeta$, and $\phi$ can be determined for every point $P$, the whole reciprocal lattice can be constructed and hence the unit cell found.



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15. But here we meet the first difficulty peculiar to fibre photographs. The direction $NS$ must be some standard direction defined with reference to the crystal structure, and we must therefore know the orientation of the crystal when it produces a given reflection. With a single crystal this is not difficult, but in a fibre we have fibrils in all orientations, and we have no means of knowing which of these fibrils produced any given reflection. Thus we can find the coordinates $\xi$ and $\zeta$ of a point $P$, but the point can still lie anywhere on a circle, as shown in Fig. 21. Each spot on the photograph defines one such circle, and

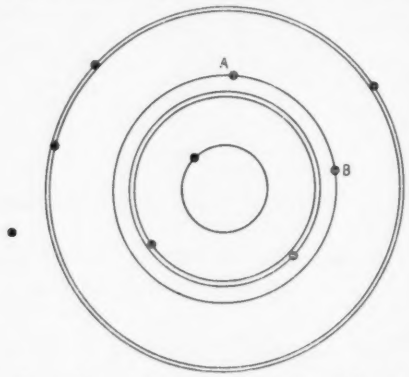


Fig. 22. The points of the reciprocal lattice lying on circles defined by the x-ray diffraction photograph.

the whole photograph therefore gives us the $\xi$ and $\zeta$ values for a series of circles such that each circle must have a reciprocal-lattice point lying on it. In general, provided the photograph has well-defined spots so that the circles are well-defined, there is only one way in which we can find a regular lattice on which to place the points. (See ref. (2), Chapter VI). Fig. 22 shows one plane of the reciprocal lattice (looking down the vertical axis in Fig. 21) with the circles through the lattice points.

16. The second difficulty that we meet in dealing with fibre photographs is that the reflections, instead of being sharp spots, are generally blurred, owing to disorientation and disorder in the fibre structure. This means that some reflections may overlap with each other so that they cannot be distinguished; this leads to ambiguity in placing them on the reciprocal lattice, as in the case of the points $A$ and $B$ in Fig. 22, which lie on two circles that are indistinguishable. Other spots may be so spread out that they become invisible on the photograph. These ambiguities can only be removed if we can improve the orientation of the fibrils, by any process suited to the material in hand.

When unit-cell dimensions have been found, it is important not only that all the observed spots on the photograph should lie in positions

corresponding to reciprocal-lattice points for this unit cell, but also that (apart from certain well-defined exceptions) there should be a spot on the photograph in every position predicted by the proposed unit cell. The exceptions are (1) spots which are absent because the symmetry of the structure (see section following) requires that their intensity shall be zero, (2) spots which happen to be so weak that they cannot be seen. In the former case the missing spots are systematically placed, e.g. they may be alternate spots along certain rows in the reciprocal lattice. In the latter case it is most improbable that the number of very weak reflections will be more than a small fraction of the total. Therefore, if many of the predicted reflections do not appear on the photograph and their absences cannot be explained in terms of symmetry of the structure, the postulated unit cell cannot be the correct one.

17. *Symmetry*

The unit cell having been found, the next step is to discover, if possible, what symmetry the structure possesses. It may be, for instance, that one half of the unit cell is a reflection of the other half in a *mirror plane*; or there may be an *axis of rotational symmetry*, so that rotation through a given angle (which may be 180°, 120°, 90° or 60°) about this axis brings the structure into coincidence with itself. There are several other sorts of symmetry, and there are in all 230 distinct *space groups*, or combinations of these symmetry elements. It is not always possible to decide to which of these space groups a structure belongs, but it is helpful if something is known about the symmetry. The important point is that symmetry considerations correlate the positions of sets of atoms, and therefore reduce the number of degrees of freedom of the structure to be determined. The symmetry can be determined partly from that of the unit cell. A structure which has, for instance, either a mirror plane or a twofold axis of rotational symmetry *must* have two of the axes of its unit cell at right-angles to the third: conversely, if the unit cell is of this shape, it is almost certain that the structure has either a mirror plane or a twofold axis. Certain types of symmetry result in some reflections being absent because the scattered wave from one atom *must* cancel the scattered wave from another atom whose position is correlated by symmetry with the first. Evidence as to symmetry can also be obtained from other physical properties: thus the occurrence of piezo-electricity or optical activity indicates the *absence* of a centre of inversion; but the absence of these properties does not necessarily show the presence of a centre of inversion.

Details of the various kinds of symmetry and their detection in crystals may be found in sources already quoted, but it must be noted that the methods readily applied to well-formed crystals are not so



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easily applied to fibres, and one may be left in some doubt as to the precise symmetry of a fibre structure.

18. *Determination of the arrangement of atoms in the unit cell*

We have already shown that the directions in which x-ray beams can be reflected depends only on the dimensions of the unit cell of the structure. It is found further that the intensities of the beams depend on the arrangement of atoms in the cell; but that the dependence is

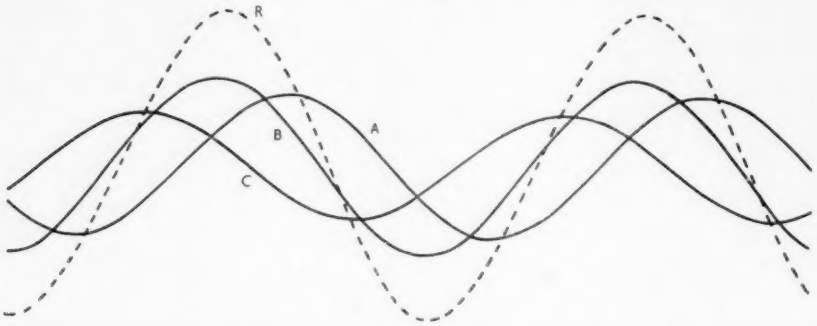


Fig. 23. The resultant of a number of sine waves.

such that it is impossible, in general, to deduce the structure directly from the x-ray intensities.

This may be explained by reference to Fig. 13. When the Bragg condition for reflection is satisfied, the waves reflected from one plane containing $A$-atoms are in phase with the waves reflected from any other plane containing $A$-atoms. Let the distance of the $B$-plane from the adjacent $A$-plane be a fraction $x$ of the periodicity $d$; then the waves reflected from the $B$-plane must lag behind those reflected from the $A$-plane by a fraction $x$ of the wavelength. The waves reflected from each set of planes may be represented by a number of sine curves, as in Fig. 23, and these will add up to give a resultant sine curve $R$, shown by the dashed line.

The intensity of the resultant beam can be calculated, since it is proportional to the square of the amplitude of the curve $R$, and in this way a standard mathematical expression is obtained [2], [4], [5], [8] for the intensity in terms of the positions of atoms.

Unfortunately there is no direct way of deducing positions of atoms from the observed intensities of reflection. We shall see later that a direct deduction could be made if we knew the phase of the resultant reflected wave, that is, the positions of the crests of the curve $R$ relative to the crests of one of the other curves, say $A$, for every reflection, but there is no generally applicable experimental method of determining

these phases. This indeterminacy of the phases is the central problem in X-ray structure determination.

19. One way out of this difficulty is to look for a structure whose calculated intensities of reflection agree (after the application of certain correction factors which need not concern us here) with the observed intensities. This is essentially a trial-and-error method, and it is necessary to start from some idea of the structure derived from sources other than X-ray diffraction. Most of this information comes from stereochemistry, for there are many well-established empirical rules which lay down the ways in which atoms can be joined together: these rules concern mainly bond lengths, bond angles, and closest distances of approach of various groups. The size of the unit cell is known from X-ray photographs, and the number of molecules to be fitted into a unit cell can be calculated if the density and molecular weight are known.

In the case of simpler compounds, there may be only one chemically plausible structure, and the only function of X-ray analysis is then to check this structure. If there are two or more distinct structures that might be possible, it is generally found that only one gives a satisfactory agreement between the calculated intensities and those observed. Or the chemical model may be a flexible structure with one or more degrees of freedom, in which case the X-ray data are used to determine which of a series of possible configurations is the best one.

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Fibre photographs, however, often show only a few distinct reflections, and care must be taken not to deduce too much from the agreement of their intensities with those of a proposed model. Clearly one could not, for instance, base a structure containing hundreds or thousands of atoms per unit cell solely on its agreement with half a dozen measured intensities.

20. *Fourier synthesis methods*

By FOURIER'S theorem, any periodic function can be expressed as a sum of sinusoidal terms. The periodic variations in scattering power in a crystal may therefore be expressed as a sum of sinusoidal, or wave-like, variations of electron density, since the electrons are responsible for the X-ray scattering. To put this mathematically, let $\rho(x, y, z)$ be the electron density at a point whose coordinates are $(x, y, z)$ and let $a$, $b$, and $c$ be the periodicities in the $x$, $y$, and $z$ directions: then by FOURIER'S theorem we can write

$$\rho(x, y, z) = \sum_h \sum_k \sum_l F(h, k, l) \cos\left[2\pi\left(\frac{hx}{a} + \frac{ky}{b} + \frac{lz}{c}\right) - \varepsilon_{hkl}\right] \quad \ldots.(8)$$

where $h$, $k$, $l$ are any three integers, and the sum is taken over all possible sets of values of these integers; $F(h, k, l)$ are constant

coefficients, and $\varepsilon_{hkl}$ are phase angles. Each of these terms represents a wave-like distribution of electron density, which, taken on its own, could diffract x-rays in one direction only. This direction satisfies BRAGG'S equation, (1), with $n = 1$; the electron-density distribution in this single term is such that there are no reflections of higher order than the first. The amplitude of the reflected x-rays is proportional to $F(h, k, l)$, and the intensity is therefore proportional to $F^2(h, k, l)$, and the phase angle of the reflected waves is equal to $\varepsilon_{hkl}$. The integers $h$, $k$, and $l$ are the *indices* of reflection.

Thus we have broken down the electron density distribution into components such that one component is responsible for each spot on the diffraction photograph. The reverse process of building up the electron-density map, by equation (8), from the x-ray diffraction data, could be performed if we could obtain the angles $\varepsilon_{hkl}$. On such an electron-density map the atoms would appear as peaks of electron density.

The phase-angle problem is somewhat simplified in the case of structures whose symmetry includes a centre of inversion: that is, if the electron density at every point $(-x, -y, -z)$ is the same as that at $(x, y, z)$. Then every $\varepsilon_{hkl}$ in equation (8) must be either 0° or 180°, or, in other words, we can omit the phase angles from equation (8) and make each $F(h, k, l)$ either positive or negative. Thus there are two possibilities for the phase of each reflection, and therefore $2^N$ possibilities for a photograph with $N$ reflections: still a very large number of alternatives.

21. Many methods have been suggested for obtaining values of phase angles so that an electron-density map can be synthesized. Not all of these are necessarily applicable to fibres, but it seems worth listing the following.

(*a*) A probable structure is taken, on chemical evidence, and the phase angles of the reflections to be expected from this structure are worked out. These phase angles are used in a synthesis in which the $F(h, k, l)$ values are obtained from the x-ray intensities. From this synthesis the likely positions of atoms are picked out, and a fresh set of phase angles are calculated: some of these will be the same as the previous set but others will be changed. With these phase angles another synthesis is performed. If the first structure was approximately correct, these successive syntheses will look more and more like a set of atoms, but if the initial structure was wrong, the electron density maps will not improve with each stage. This process is one example of *refinement* of a structure.

(*b*) If the unit of structure contains a heavy atom (one of high atomic number and therefore high scattering power), or if heavy atoms may be incorporated chemically into the structure, then we assume

that this heavy atom, to a first approximation, "swamps" the effect of the rest of the structure, and that the phases of all the reflections must be the same as they would be if only the heavy atoms were present. Therefore these phases are used in making a synthesis, which may subsequently be refined as described above.

(*c*) Recently, a number of "phase-limiting" relations have been found.[19] These are sets of relations involving $F$'s and $\varepsilon$'s which must be satisfied if the synthesis in equation (8) is to represent a structure: that is, if it is to have peaks of electron density separated by regions of low density, and positive electron density everywhere. These relations may be sufficient, in rare cases, to determine all the phases, but generally they can only limit the number of possible phase combinations to a reasonably small figure. It may then be feasible to try each combination of phases in a synthesis to find which gives the most reasonable structure.

22. The above considerations apply to crystal structure determination in general, but their application to fibres may be made difficult because of the diffuseness of the spots and the inevitable uncertainty of the angle $\phi$ to be associated with each spot, as explained in section 15. The diffuseness of the reflections leads to overlapping of the spots and consequent difficulty in deciding how the intensity should be partitioned between the reflections. Thus it is essential to use every possible means of making the spots sharp, by improving the order in the specimen. The lack of knowledge of the angle $\phi$ means that the positions of spots in the reciprocal lattice can only be fixed if a unit cell can be found, and even then there may be some doubt as to how the intensity ought to be divided between two distinct reflections whose spots overlap on the photograph.

If the spots are so diffuse that it is impossible to find a unit cell with any certainty, and a structure cannot be found by the usual methods, we must consider how much can be deduced from the fibre diagram. We have seen already in sections 5–11 how some general features of the fibre structure may be obtained. It may be possible to derive some further information from another type of synthesis, the Patterson diagram, which can be made without any knowledge of phase angles.

23. The *Patterson diagram* is obtained from the synthesis

$$P(x, y, z) = \sum_h \sum_k \sum_l [F(h, k, l)]^2 \cos 2\pi \left(\frac{hx}{a} + \frac{ky}{b} + \frac{lz}{c}\right) \quad \ldots . (9)$$

and it can be shown that it represents a map of vector distances between atoms. Thus a peak at $(x, y, z)$ in the Patterson diagram implies that somewhere in the structure there are two atoms whose separation is $(x, y, z)$, and there will be one peak on the Patterson

diagram corresponding to every such separation between a pair of atoms. Fig. 24 shows the Patterson diagram corresponding to the set of four atoms $A$, $B$, $C$, $D$. The point $AB$ on the Patterson diagram represents the vector distance between atoms $A$ and $B$. But in practice we are given only the peaks in the diagrams without any enlightenment as to what pair of atoms is represented by each peak. Several methods have been devised by which it should be possible, at least ideally, to disentangle this set of interatomic distances so as to find the original structure.[20] But the main difficulty is that a unit cell containing $N$ atoms will give a Patterson diagram with $N(N-1)$ peaks, and if $N$ is

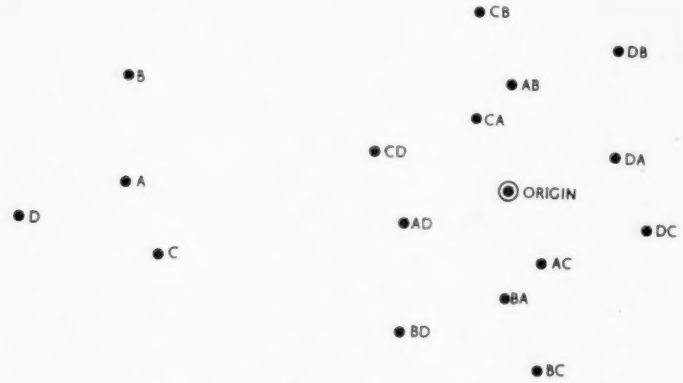


Fig. 24. Four atoms and their Patterson diagram. (See text.)

a large number, these peaks will be so numerous that they will be hopelessly confused.

If the fibre diagram is very diffuse, we cannot separate out the values of $F(h, k, l)$, but we can still use a modified Patterson synthesis devised by MACGILLAVRY and BRUINS.[21] This is equivalent to a Patterson diagram which has been rotated about the vertical axis (the fibre axis) and an average taken. Thus it gives us the length and the inclination to the vertical of each interatomic vector, but its direction is otherwise indeterminate.

From this diagram it is possible to pick out some features of the structure. An instance of its use has been given by FRANKLIN and GOSLING,[22] in connection with the X-ray investigation of nucleic acid. They were able to derive the way in which the chains were packed side by side in the fibre, and also showed that a series of peaks lying on a curve were consistent with a two-chain helical configuration in the structure. It should be noted, however, that these features can be deduced directly from the diffraction photograph.[23] The Patterson diagram contains no more, and no less, information than the X-ray

photograph, but the information is presented in a way which may make the interpretation in terms of structural models rather easier.

The number of atoms in a unit cell of a substance like nucleic acid is so large that the peaks on the Patterson diagram are far too numerous to be seen separately. Any interpretation must therefore be in terms of groups of atoms rather than individual atoms.

24. *Helical structures*

The idea that some long-chain biological materials, such as proteins, nucleic acids, etc., might be built up in a helical configuration arose both

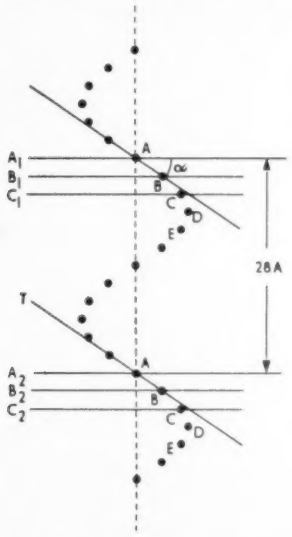


Fig. 25. Some reflecting planes for atoms on a helix.

from stereochemical considerations[25] and from the fact that the general character of the x-ray diffraction photographs suggests this possibility. The diffraction photograph of deoxypentose nucleic acid (D.N.A.), for instance, shows empty regions above and below the centre of the pattern (Fig. 8, between pages 140–1). The layer-lines (see section 7) indicate a repeat-distance in the vertical direction of 28 Å, but there is no sign of spots corresponding to this repeat-distance *on* the meridian. If the 28-Å repeat were due to a strongly-scattering group of atoms occurring at every 28 Å along the axis of the fibril, it ought to show up as a repeat on the meridian, as explained in section 6.

Consider now a row of points arranged along a helix of pitch 28 Å as shown in Fig. 25. The vertical repeat-distance of this structure is then the pitch of the helix. Let many such helices be packed side by side. Let us consider the reflections from a set of planes spaced at 28 Å



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perpendicular to the helix axis, shown by $A_1$, $A_2$, etc. in Fig. 25; all these planes will pass through similarly-situated atoms $A$. Now, as in section 18, draw parallel planes through all the other atoms, which we again denote by $B$, $C$, $D$ . . ., and consider the reflections from all these planes. In the first-order reflection ($n = 1$ in equation (1) ) the waves reflected from $A_2$, are just one wavelength behind those reflected from $A_1$, and hence the waves reflected from the planes $B$, $C$, $D$ . . . are respectively $1/m$, $2/m$, $3/m$ . . . wavelength behind those from $A_1$, where $m$ is the number of points on one turn of the helix. The

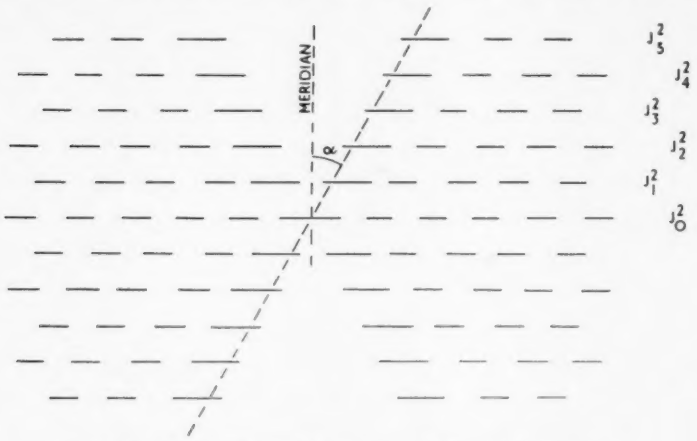


Fig. 26. The maxima of the squares of Bessel functions.

resultant wave has zero amplitude, because all these reflected waves cancel each other out. The same applies to the second order reflection, for which the reflected waves from $B$, $C$, $D$ . . ., will lag behind those from $A$ by $2/m$, $4/m$, $6/m$ . . . wavelength. In fact, all orders on the meridian will be of zero amplitude until we reach the $m^{\text{th}}$ order, for which all the planes reflect in phase, and we have a strong reflection.

A helical structure will therefore account for the absence of spots on the meridian of the photograph. A detailed treatment of the diffraction properties of a helix[24] shows that a *continuous* helix (or helical "wire") would give a pattern in which the intensity on the $n^{\text{th}}$ layer line varies as

$$[J_n(2\pi r\xi)]^2 \qquad \ldots.(10)$$

where $J_n$ is the $n^{\text{th}}$-order Bessel function of the first kind, $r$ is the radius of the helix, and $\xi$ is the coordinate in reciprocal space defined in section 14 (Fig. 21) and is approximately proportional to the distance of the spot from the meridian. The appearance of this intensity distribution is shown in Fig. 26, in which the regions of largest intensity of the Bessel functions have been plotted.

It is further shown[24] that if the helix is broken up into a series of points, as in Fig. 25, the central part of the pattern is unchanged, but there are also superimposed on it further patterns, identical with the first, but with their origins shifted to points on the meridian on the $m^{\text{th}}$ layer lines above and below the centre, also on the $2m^{\text{th}}$, $3m^{\text{th}}$ . . . layer lines. This gives rise to a resultant pattern with empty diamond-shaped regions above and below the centre. Such a pattern is shown in Fig. 28 (*b*), which is an optical diffraction pattern of a projection of a helical structure.

From the properties of Bessel functions we can say that the angle between the sides of this diamond and the meridian will be equal to the angle $\alpha$ between the coils of the helix and the plane normal to the helix axis, or pitch angle of the helix. If we draw a set of planes tangential to the turns of the helix—the planes $T$ in Fig. 25—we can see that there is a concentration of atoms on or very near these planes, so we expect all orders of reflection from these planes to be strong. The planes make an angle $\alpha$ with the horizontal, and the row of reflections produced by them will therefore make an angle $\alpha$ with the vertical. This is one of the rows of reflections bounding the empty diamond-shaped region.

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The diffraction pattern of D.N.A. is sufficiently like the pattern in Fig. 28 to afford a strong indication that D.N.A. has a helical structure. Another point of similarity between the two patterns is the nearly periodic rise and fall of intensity along each layer line. It is not possible to say with certainty that another type of structure could not be found to give the same kind of diffraction pattern, but this seems unlikely.

Some features of the helix can be derived directly from the photograph. The layer-line spacing gives the pitch of the helix; the angle between the sides of the diamond and the meridian gives the pitch angle, and the number $m$ of atoms (or more generally groups of atoms) per turn of the helix is given by the number of the layer line on which the first meridional reflection appears. It may be noted here that $m$ need not be an integer; the patterns with their origins above and below the centre will then form separate sets of layer lines, not superimposed on the layer lines of the central pattern. The diameter of the helix may be obtained from the pitch and the pitch angle, or it may be found from the periodicity of the rise and fall of intensity along the layer lines.

25. The above analysis applies only to a single set of points arranged along a helix. A *double helix*, or two helices intertwined, would give a similar pattern except that all the odd-order layer lines would have zero intensity, and the remaining even-order layer lines would have intensity distributions varying as the squares of even-order Bessel functions. The layer lines are now twice as far apart, corresponding to the fact that the repeat-distance along the structure is half the pitch.

Similarly three intertwined helices would give only every third layer line, and so on.

We can proceed from this simple model to a real structure containing groups of atoms arranged helically by noting that each atom of the group will lie on a helix with corresponding atoms of the other groups, and adding up the Bessel functions for all these helices. The reverse process, of deducing the arrangement of atoms in the groups from the distribution of intensity along the layer lines, would be more useful for structure determination, but is much more difficult because of the inconvenient mathematical properties of Bessel functions. This process has been attempted by WILKINS *et al.*,[23] who have shown that most of the X-ray photograph of D.N.A. can be interpreted in terms of one double helix and another smaller helix, coaxial with the first: this is broadly in agreement with a structure proposed by WATSON and CRICK[26] on stereochemical grounds.

### 26. *Optical diffraction methods in structure determination*

The calculations of intensities of diffraction from proposed structures is a tedious process, and much time can be saved, as BRAGG first pointed out[27] by the use of an optical analogue. If a periodic structure is projected on to a plane, a two-dimensional periodic pattern will result, and if this pattern (reproduced on a suitable scale) is used as a diffraction grating for parallel light, the diffraction pattern so obtained is closely related to the X-ray diffraction pattern of the structure. The intensities of the diffracted light-beams are in fact proportional to the intensities of those X-ray reflections represented by points in the reciprocal lattice lying on one plane; the plane through the origin parallel to the plane on which the crystal structure has been projected.

This forms the basis for a rapid method of obtaining the intensities of diffraction for any proposed structure. It is not claimed, of course, that the accuracy of this method is comparable with that obtained by calculation, but it can be very useful in the initial trial-and-error stages of a structure determination.

The diffraction gratings are readily made by punching holes in thin card or in photographic film, a pantograph being used to reduce the pattern to a scale of about 2 mm per Ångstrom unit. The resulting mask is placed in a diffraction spectrometer, shown diagrammatically in Fig. 27. Parallel light falls on the mask, and the diffracted beams are brought to a series of foci by a lens of long focal length, and the diffraction patterns can be viewed by means of a low-power microscope. They can also be photographed by attaching a camera to the microscope. The apparatus is described by HUGHES and TAYLOR,[28] and the possibilities of optical diffraction methods have been described by LIPSON and others.[29]

In practice the pattern need not be repeated many times on the mask; the same information can be obtained by using a mask with a single unit of the pattern or with four units representing four adjacent unit cells of the crystal structure.[30]

This procedure gives us the diffraction pattern for a single projection of the crystal structure. But in order to simulate a fibre photograph we must superimpose diffraction patterns of projections on to all planes parallel to the fibre axis. This means that we must make up a

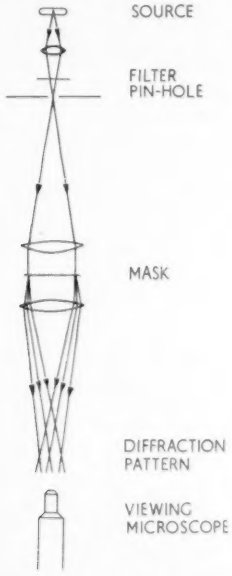


Fig. 27. The diffraction spectrometer.

number of masks with projections of the proposed structure on to a series of planes, all parallel to the fibre axis and with equal angular spacing between them. The diffraction patterns of these must then be superimposed. This may be done photographically by placing the masks in the diffraction apparatus in succession and exposing one film to all the diffraction patterns. This method does not permit one to view the resultant pattern directly, and an alternative is to punch all the projections on the same mask, as far as possible from each other, with their fibre axes parallel, and to view the diffraction pattern of this mask. This suffers from two disadvantages: that there is less room available on the mask for each projection, and that the resulting diffraction pattern is not truly the sum of the intensities of the diffraction patterns of the projections but shows some effects due to interference between the different projections. This interference is not serious, however, if the projections can be well spaced apart on the mask.

A further question arises, as to how many different projections of the structure we must use in order to simulate correctly a fibre photograph in which the fibrils take, in effect, an infinite number of orientations. This has been considered by the author[31]; it is shown that the required number of projections depends on the range of periodicities to be covered, and that if periodicities from the longest down to some value $d_0$ are to be covered, the angle between the planes of projection must be not greater than $d_0/D$ radian, where $D$ is the diameter of the unit of structure in the fibre. Thus if the fibre is built up of chains of overall diameter 20 Å, and it is required to reproduce all reflections with periodicities greater than 2·5 Å, the planes on which the structure is projected must be spaced at less than 2·5/20 radian, or about $7\frac{1}{2}°$; an interval of 6° would probably be safer.

In the case of helical structures, the number of projections can be much smaller. This is because the projection of a helical structure is unaltered if the plane of the projection is turned through an angle of $\frac{360°}{m}$ about the fibre axis, where $m$ is the number of identical groups of atoms per turn of the helix. Thus the angle between successive projections is still calculated in the same way, but the angular range to be covered is smaller. In the same way, some symmetry elements can reduce the required number of projections by making pairs, or larger groups, of projections identical. Fig. 28 shows the optical diffraction pattern of atoms equally spaced along a helix.

27. A limit is set to the size and complexity of the unit cell of a structure that can be investigated by optical methods, by the fact that the size of the mask is limited to the aberration-free area of the lenses of the diffraction spectrometer, and by the fact that the holes representing the atoms cannot be made smaller than the smallest satisfactory punch. The holes may not overlap, so there is a limit to the number of atoms that can be represented. When a complex structure is projected on to a number of differently-oriented planes, some overlapping of the projected atoms is very likely to occur. If an occasional overlap occurs, it seems legitimate to push the overlapping "atoms" apart so that they only touch each other, but if there are many overlaps, this cannot be done without risk of altering the resulting diffraction pattern considerably.

Thus the application of the optical diffraction analogy to the interpretation of fibre diagrams is not quite so simple and direct as its application to simpler structures, for which a single projection is often sufficient. But the calculation of the intensities of reflection for a complex fibre structure is correspondingly more tedious than the calculation for a simple crystal structure, and the use of optical methods can therefore be of considerable value in selecting probable structures and rejecting others.

(*a*)

Fig. 28. (*a*) Two projections of a row of atoms on a helix.
(*b*) Superimposed optical diffraction patterns of (*a*).

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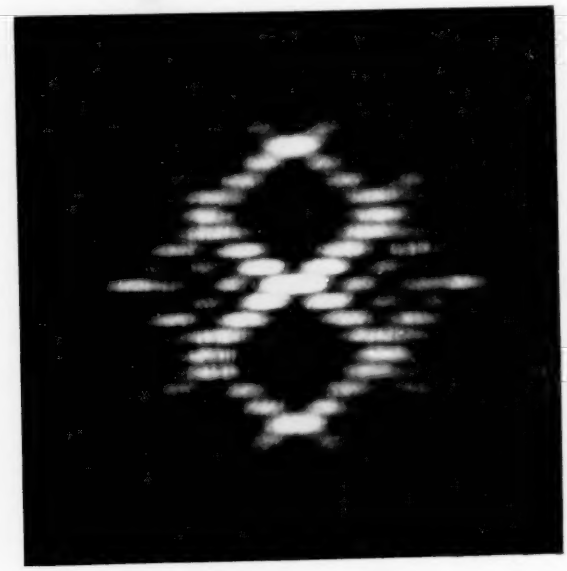

(*b*)

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# RECENT STUDIES ON THE REVERSIBLE DENATURATION OF PROTEINS

*M. Joly*

## Introduction

Although a considerable amount of work has accumulated during the last fifty years on protein denaturation, it is nevertheless still difficult to enunciate a precise definition of what is commonly called denaturation, and to give a description which would be both general and accurate. Indeed, on account of the great number of parameters which is involved in protein studies, large discrepancies frequently occur between the results found in the literature, these differences being due to the fact that the experimental conditions were not exactly the same in different experiments.

If a rigorous discrimination is made between the well-established experimental facts and the more or less consistent assumptions which have been introduced in the different tentative interpretations, it appears that many problems concerning protein denaturation are yet unsolved. The following questions, for instance, remain to be answered: Is denaturation a "unique" molecular process, the same for all proteins and with all denaturing agents, or are there several types of denaturation, depending on the system studied? Is denaturation an "all-or-nothing" phenomenon, or can different stages be observed during the protein modification? Is the protein transformation, described as denaturation, a continuous, a stepwise or a discontinuous process? Is, in certain well-defined cases, denaturation a true reversible phenomenon or is the often observed reversal only apparent? Moreover, is the idea of denaturation consistent with the concept of reversibility? On the molecular scale, does denaturation proceed by an entirely intramolecular mechanism or, sometimes, by a partly intermolecular one?

Our aim in the present article, as indicated by its title, is not to summarize the numerous publications on protein denaturation (good reviews for that purpose have already been published by various authors [81], [113], [124], [201]), but to discuss in detail the question of the reversibility of denaturation. It seems impossible, however, to separate the study of the reversal of denaturation both from the general picture of protein denaturation and from a discussion of the problems listed above.

In the following study we shall try to distinguish the phenomenological aspect of protein denaturation and its reversal—that is to say,

the description of the facts observed in well-defined experiments—from the interpretations suggested by current speculations on protein structure and chemistry to explain the transformation of the protein molecules from the native to the denatured state or vice versa. Indeed, although it is now possible to give an almost completely satisfactory picture of the general molecular process of protein denaturation (whether reversible or not), it is very important not to forget that this interpretation includes several assumptions for which more proofs or demonstrations are still needed.

After briefly discussing what is meant by denaturation and to what extent one or several types of denaturation can be distinguished, we shall sum up the phenomena of denaturation in protein solutions, and describe the principal experiments relevant to the process of denaturation by different denaturing agents and to its reversal. The properties by which denatured and reversed proteins can be characterized with respect to native proteins will be summarized. Then the experimental data supporting the idea of reversible denaturation will be discussed in detail and the corresponding equilibria examined from the standpoint of kinetics and also in connection with partly reversible denaturation and incomplete reversal of the denaturation. Particular attention will be paid to "inhibitors" of denaturation and to the importance of aggregation processes in the secondary effects of denaturation. Thus it will be shown, for instance, that the irreversibility of denaturation is frequently due to the aggregation of the denatured protein molecules, and that the appearance of the $\beta$-structure seems to be closely related to the possibility of aggregation. The intra- and inter-molecular processes involved in denaturation (reversible or not) will be studied and, as a conclusion, a tentative general picture of the structural and energetic mechanism of the protein denaturation will be proposed, as illustrated by recent works on the subject.

In the present paper we shall restrict our study to the denaturation of proteins in solution, to the exclusion of denaturation of other substances like nucleic acids or nucleoproteins (virus proteins, etc.). Our investigation will also exclude what is often called surface denaturation, because this phenomenon cannot be seriously discussed without a complete examination of the difficult questions presented by protein monolayers; such a discussion would go far beyond the limits of this article. For a similar reason denaturation by ultra-sonic or electromagnetic waves will not be taken into consideration.

## Definition of Protein Denaturation

Biochemists commonly speak about the "denaturation" of proteins and everybody understands what is meant, although it is very difficult to define precisely its meaning or to decide to what extent certain



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phenomena can be included. Thus, according to LANGMUIR, denaturation is something "essentially vague,"[58] whereas, according to MIRSKY and ANSON, denaturation is a definite chemical reaction.[48]

As frequently emphasized, the proteins possess the characteristic of undergoing profound changes in many specific properties when subjected to treatments so mild that ordinary compounds are unaltered. Such modifications of proteins are ordinarily called denaturation; they correspond to changes just sufficient to cause the protein to lose its identity, i.e. certain specific properties.[58] Most commonly a protein is said to be denatured when it is insoluble in a medium in which it is soluble while still native.[83]

Each protein, however, has a large number of specific properties, and we see immediately that the choice of any one property as a criterion of denaturation is arbitrary and that, according to the chosen characteristic property, a given protein may be classified denatured or native; according to one criterion denaturation has occurred, while according to the other it has not.[58] For instance, is the transformation of egg albumin, by ageing at room temperature, a denaturation or not? The two forms are soluble at the isoelectric point and easily crystallizable; they have the same sedimentation constant, but different diffusion constants and electrophoretic mobilities.[111], [112]

Denaturation is therefore often described in terms of simultaneous changes in several properties.[58] Sometimes a more restrictive definition is given and by denaturation is meant the loss of one or more of the most characteristic properties of a given protein[116]; the difficulty remains, however, in deciding what properties are most characteristic. Occasionally it has been proposed to characterize denaturation as a transformation occurring with an extremely high entropy and heat of reaction or activation energy,[116] but such a definition is not a purely phenomenological one; it implies assumptions on the molecular process and kinetics of the denaturation, and this is perhaps not general enough. It seems to be preferable to take as a definition of denaturation the change or loss, by mild treatment, of any specific property of the particular protein, the simultaneous loss of several properties appearing as different aspects of the same underlying process. More drastic modifications of the protein molecules, for instance those accompanied by release of amino acids or peptides, are no longer denaturation; thus the transformation of egg albumin into plak-albumin by enzymatic action is not a denaturation.

Two questions are now to be answered about the "all or nothing" character of the denaturation and about the existence of only one or of several kinds of denaturation.

Some authors consider that a protein molecule is either wholly native or wholly denatured.[42] Others think that different degrees of

denaturation should be taken into account[58] and that partly denatured protein is not necessarily a mixture of native and denatured molecules. According to this idea, the changes taking place in the molecule on denaturation may be many and varied, the same molecular structure undergoing denaturation in somewhat different ways, so that in general a denatured protein is not a definite substance but rather is a mixture of many products.[58], [116]

In support of the first view, urea-denatured egg albumin behaves in presence of $K_3Fe(CN)_6$ as a mixture: the protein portion that is not precipitated at the isoelectric point does not differ from the native protein in SH content; the insoluble portion is completely devoid of SH; no intermediate products can be detected. Likewise, serum albumin partly denatured by urea shows two fractions in electrophoresis: native and denatured.[223] Supporting the second point of view, it can be quoted that in the partial recovery by compression of heat-denatured egg albumin the solubility is completely restored whereas certain other properties are not fully regenerated.[222]

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The uncertainty of the "all or nothing" character of the denaturation, is perhaps related to the difficulty of deciding whether denaturation is the same process for a given protein whatever the denaturing agent may be. Several authors have shown the identity of denaturation by various denaturing agents, such as heat and dilute acids or alkalies:[6] for instance, salicylate and heat in the case of haemoglobin,[32] or heat and pressure in the case of pepsin and rennin.[73] Similarly the urea denaturation of fibrin does not differ from that of fibrinogen.

The opposite opinion is more frequent and probably more reliable. For instance, although a certain analogy between the properties of denatured serum albumin and denatured egg albumin is apparent, the analogy is by no means complete.[102] Fibrinogen, which is soluble at urea concentrations higher than 30 per cent, behaves differently from egg albumin, serum globulins or serum albumin, which form gels even in urea solutions as concentrated as 60 per cent.[191] With regard to the biuret reaction of heat-denatured proteins, egg albumin, serum albumin, euglobulin and pseudoglobulin show the same behaviour, although casein does not.[202]

In view of the great diversity of properties of the various proteins, it is not surprising that after denaturation their properties are still different, even if denaturation corresponds to a decrease in the protein specificity.

The similarity of the denaturation of a given protein, under the action of different denaturing agents, also appears disproved. Indeed, since in the case of serum albumin, aggregation occurs following cooling of the heat-treated solution, no evidence can be adduced that after heating the denatured serum albumin molecule is comparable in size and shape

with that in the presence of urea or guanidine hydrochloride.[102] Moreover, by comparison of heat-denatured and pressure-denatured serum albumin, it appears clearly that the products have many different properties,[239], [252] as we shall see later. Likewise, denaturation by urea and guanidine does not influence the antigenic specificity of serum albumin,[99] while heat, alkali and acid denaturation cause a marked alteration of the specificity of egg albumin, although all the denatured proteins react fully with antisera produced against the differently denatured proteins.[123]

We shall be able to give more accurate conclusions only after describing the fundamental denaturation phenomena and after discussing the molecular mechanism of denaturation.

### Macroscopic Changes Characterizing Denaturation

*Solubility.* Several properties of protein solutions are modified during denaturation: firstly, the loss of solubility is generally chosen as a criterion. This phenomenon is so well known that it is needless to insist on it,[23], [48], [52], [88], [117] but one can perhaps remember that two kinds of solubility must be considered; the solubility in the presence of the denaturing agent and the solubility in the absence of the denaturing agent, e.g. in water at the isoelectric point or in a given electrolyte solution, both before and after denaturation has occurred. We shall discusss later the molecular significance of this loss of solubility. Let us now give a few examples showing how the loss of solubility depends on the method of denaturation. Thus the solubility may decrease with increasing temperature or time of heat treatment, as for example with casein,[158] whereas this protein is still completely soluble in 0·01 *N* and 0·1*N Na OH*. Similarly the solubility is differently affected by heat and by high pressure treatment. Thus, for horse serum albumin, the percentage precipitation, as measured after 1 hour at 22°C, at *p*H 4·7 in acetate buffer and $Na_2SO_4$ ($\mu = 2{\cdot}78$), has the following values;[251] the 3 per cent protein solution has been heated 10 minutes at 65°C under atmospheric pressure:

| *p*H: | 3·7 | 4 | 4·25 | 4·5 | 6·9 | 7·5 | 7·7 | 8·3 |
|---|---|---|---|---|---|---|---|---|
| Per cent precipitation: | 2 | 54 | 92 | 100 | 90 | 89 | 90 | 88 |

whereas, if a 6 per cent solution has been compressed 16 hours at 37°C under 10,000 kg/cm², the following values are found:

| *p*H: | 3·7 | 4 | 4·25 | 4·5 | 4·6 | 6·9 | 7·5 | 7·7 |
|---|---|---|---|---|---|---|---|---|
| Per cent precipitation: | 39 | 0 | 0 | 0 | 1·5 | 91 | 88 | 87 |

*Viscosity.* The increase of viscosity is also frequently chosen as a criterion of denaturation[25], [72], [88], [89], 103), [158], [203] in consequence of the ease with which such measurements can be made. But in spite

of the large amplitude of the viscosity variations, this criterion is not very accurate, because the viscosity is very sensitive to secondary effects of denaturation such as aggregation or changes in interaction rather than to the denaturation itself. Moreover, the values of the viscosity found for a given protein denatured by a given denaturing agent are not specific. Thus the viscosity may vary by ageing the denatured protein.[122] It depends sometimes on the concentration of the denaturing agent; for instance, the urea denaturation of fibrinogen is accompanied by an increase of the viscosity of the solution if the urea concentration is below 20 per cent (the viscosity of denatured fibrinogen is about double than of native), but at 30 per cent urea concentration the viscosity does not change.[191] With certain proteins the denaturation does not induce a viscosity increase; for instance in the weak denaturation of arachin, amandin and Bence-Jones protein, the viscosity does not change.[217] Similarly, with serum albumin, heating at *p*H 3, where no aggregation takes place, produces practically no change in the viscosity in the presence or absence of salt, and even at *p*H 9·7 only an insignificant rise occurs.[245] In view of these anomalies, the interpretation on the molecular scale of the viscosity data is quite difficult, as we shall see later.

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*Double refraction of flow.* Changes in streaming birefringence also give information about the denaturation process, chiefly for very elongated particles or when aggregation and denaturation occur at the same time. Thus, in the case of myosin, denaturation by chemical agents is shown by rapidly diminishing double refraction of flow;[72] on the other hand in the heat treatment of egg albumin[137], [208], [209], [213] and of serum albumin[166], [181], [184], [200], [239], [252] denaturation and concomitant aggregation are manifested by the appearance of streaming birefringence.

*Turbidity.* An increase of the turbidity frequently parallels the increase of viscosity[122] and is used as a measure of denaturation[24], [110], [172], [291], [220], [230] It is, however, generally due to aggregation of the particles.

*Polarography.* The polarographic behaviour of proteins may be modified by denaturation. For instance, compared with native egg albumin, alkali and urea denatured egg albumin give higher waves.[235]

*Electrical behaviour.* A shift of the isoelectric point is shown by several denatured proteins. Thus, heat denatured serum albumin has an isoelectric point 0·2–0·5 units more alkaline than the native material.[24] Such a phenomenon is to be related to the deformation of the titration curves. For instance, a *p*H shift is generally observed on heating egg albumin solutions; this shift is 0·1 *p*H unit or less on the acid side of the isoelectric point and in the upward direction; above the isoelectric point the *p*H is shifted towards 8·0, the magnitude of the shift increasing

as the *p*H of 8 is approached from either side.[220] Likewise the titration curves of carboxyhaemoglobin are characterized by an anomalously high increase in bound acid between *p*H 4 and 3; haemoglobin rapidly denatures in this region and back-titration yields an entirely different curve; changes in the increase in bound acid are parallel, at each *p*H, to the concomitant changes characteristic of denaturation. Indeed, there is exact correspondence between the delayed acid uptake and the fraction of total haemoglobin which is denatured.[205] In connection with this we may note the following curious effect: if unstretched fibres of pure wool are stretched by 50 per cent, then immersed for 90 seconds in boiling water, unloaded and kept in hot water 8 minutes, and are finally titrated with 0·01 N HCl, it is found that, while the isoelectric point of normal wool is *p*H 5·5, that of the stretched product is 5·29, and that of shrunk wool is 5·8. Thus stretching causes a shift to the acid side, while shrinkage causes a shift to the alkaline side.[229]

The electrophoretic behaviour of proteins is also often altered by denaturation. In 20 per cent urea solution, urea-denatured fibrinogen is electrophoretically homogeneous and its mobility does not differ from that of native fibrinogen;[191] on the other hand, the *p*H-mobility curves of native and heat denatured serum albumin are only approximately parallel, although heat denatured serum albumin is electrophoretically homogeneous.[24] At *p*H 3·6 ($\mu = 0{\cdot}1$) the electrophoretic mobility of the heat-treated horse serum albumin is about 20 per cent higher than that of the unheated material; at *p*H 7·6 the denatured protein migrates with a single boundary in a buffer of 0·02 ionic strength, but with a double boundary in buffers of higher ionic strength.[102]

*Optical activity.* Important changes in optical rotation are frequently characteristic of denaturation. For instance, a progressive urea-denaturation of $\beta$-lactoglobulin is accompanied by a considerable increase in laevorotation.[168] In the denaturation of $\gamma$ globulin with guanidine hydrochloride of strength 1·5 M, or of chymotrypsinogen with 2 M, or of lysozyme with 4 M, the optical rotation increases with time until constant values are reached; a further increase in laevorotation takes place upon heating with guanidine hydrochloride at 50 or 55°C.[221] In the case of legumin or tuberin 5 M $HN{:}C(NH_2)_2$—2HCl increases optical rotation considerably more than does salicylate or urea.[203]

*Volume changes.* For some proteins denaturation may be accompanied by a considerable change in volume. Thus, upon slow denaturation by alkali at 30°C and *p*H 8·5 $\beta$-lactoglobulin shows a volume change of — 200 ml. per mole;[173] a volume contraction of about — 250 ml/mole is also observed in the denaturation by urea, the rate of variation being much greater at 0°C than at 30°C.[144] On the other hand the effect of pressure on the heat denaturation of human

serum-globulin seems to indicate a volume increase for activation in the denaturation reaction of the order of 100 $cm^3$ per mole.[138]

*Enzymatic behaviour.* When the protein under examination is an enzyme, denaturation causes more or less inactivation. Thus the activity of pepsin and rennin decreases with pressure increase for constant exposure time and is completely destroyed at pressures ranging between 5000 and 6000 $kg/cm^2$; activity decreases also with increase of exposure time at constant pressure, but to a lesser degree, and depends on temperature, *p*H and ionic strength.[73] On the contrary, neither trypsin nor chrymotrypsin is much affected by pressure at *p*H 4 and below; but above *p*H 4 inactivation increases with increase in *p*H to a maximum at about *p*H 7·6. For each enzyme, at a constant *p*H, concentration and period under pressure, there is a minimum pressure below which little or no inactivation occurs; increase in pressure above this point results in an increase in activation up to a maximum. Further increase in either the pressure or in the time of action has little or no effect, but multiple application of pressure results in a considerable increase in inactivation above that obtained at the same pressure and for the same time in a single application. Trypsin is less readily inactivated than chymotrypsin.[187]



*Immunological behaviour.* Differences between native and denatured proteins have been also checked from the standpoint of immunochemistry, despite the difficulties in interpreting immunological reactions when solubility, state of aggregation and specific patterns of the reacting particles may be simultaneously modified. As emphasized by MacPherson and Heidelberger,[121] little attention has been paid to the state of aggregation or degradation, factors which not only are expected to influence the immunological properties but which also, if neglected, cause confusion as to the changes attributable to denaturation alone. Thus alkali-denatured egg albumin, in reaction with excess antigen shows a higher antibody-antigen ratio than does acid or heat-denatured egg albumin. However, within any one type of change the results may be modified by aggregation: decrease in the size of denatured egg albumin aggregates lessens the amount of nitrogen precipitated from anti-egg albumin serum.[123] The complexity of such a phenomenon explains the discrepancies sometimes found between the conclusions given by different authors. For instance, according to Wu and collaborators[11] and to Haurowitz and Bursa[125], egg albumin denatured by heat reacts with anti native egg albumin serum; but according to Grabar and Kaminski[183] this effect is due only to impurities like conalbumin and ovomucoid, since after treatment of the serum with these substances, heat denatured egg albumin no longer reacts with the anti native egg albumin serum. Furthermore, immunological reactions seem to be rather insensitive for the study of

denaturation, probably because of the importance of secondary effects. Thus in the case of antipneumococcal horse serum globulin denatured by guanidine hydrochloride, quantitative precipitations, performed on rabbit antisera with native and irreversibly denatured horse antibody and normal globulin respectively, show no significant differences in the antigenic activity of the proteins. Their cross-reactivity leads to the conclusion that the native and irreversibly denatured fractions of antibody globulin are antigenically more closely related to each other than the corresponding fractions of normal globulin and vice versa.[115]

*Chemical behaviour.* Protein denaturation is accompanied by profound changes in the chemical behaviour of the protein molecule. One of the most characteristic chemical changes is the variation in the number of detectable SH groups. For instance, in native egg albumin no SH groups are detectable, whereas in completely coagulated egg albumin as many groups are detectable as are found in the hydrolysed protein;[42] in the case of pressure-denatured egg albumin, the exposure of SH linkages increases with the magnitude of the pressure.[82] Roughly speaking, the effect of denaturation on the SH groups of a protein is to extend towards the acid side the *p*H range of their activity.[43] Exhaustive investigations have been carried out on the behaviour of SH groups in protein denaturation[17], [18], [23], [35], [45], [46], [59], [62], [64], [71], [72], [234], [235]. The number of detectable SH groups depends on the denaturing agent; for instance, the number of SH groups found in heat denatured egg albumin is less than that found in egg albumin in concentrated urea, but it is the same as that found in urea-denatured egg albumin solution after diluting.[79] Nevertheless the exposure of SH groups seems to be an "all-or-nothing" process on the molecular scale; indeed in partially heat-coagulated egg albumin the soluble fraction contains no detectable groups and the insoluble fraction contains the number found after hydrolysis.[42] As denaturation proceeds in urea, SH groups are liberated only in the egg albumin with altered solubility when the maximum number of SH groups is liberated;[83] thus in a molecule of egg albumin either all or none of its SH groups that give a test with nitroprusside are liberated.

The activity of reducing groups other than SH is also enhanced by protein denaturation.[44] There are similar changes in S-S bonds and phenolic groups of tyrosine.[79], [147] The reactivity of amino groups is likewise involved in denaturation process; thus 12 of the 31 lysine $\varepsilon$-$NH_2$ groups per molecule of $\beta$-lactoglobulin do not react with 1:2:4 fluorodinitrobenzene unless the protein is first denatured; one-half to one-third of the lysine $\varepsilon$-$NH_2$ groups of native serumglobulin do not react, and in horse pseudo-globulin a similar number fails to react with ketene or acetic anhydride. However, all the groups become reactive to all the reagents after denaturation, and each type of denaturation

exposes all the amino groups.[147] Chemical behaviour of peptide linkages is also modified after denaturation,[79] when they show enhanced activation. For instance, with denatured egg albumin, the biuret reaction becomes more rapid than for the native protein.[170], [202]

Closely related to such chemical effects of denaturation is the increase in the susceptibility of the protein to enzymic digestion induced by denaturation.[52] For instance, at *p*H 7·6 the heat-treated horse serum albumin is more easily digested by trypsin than is the native form.[102] Likewise $\beta$-lactoglobulin is much more easily split by trypsin when a small quantity of urea is added,[168] and urea denaturation markedly increases the rate of papain digestion of human serum albumin.[118] Frequently denaturation seems to be the initial step of proteolysis;[76] thus, insulin, which is difficult to denature, is split by chymotrypsin but not by trypsin.[143]

*Binding ability.* A property which characterizes the proteins is their tendency to associate with other organic compounds. Frequently denaturation of the proteins causes large changes in the binding powers. For instance, the binding capacity of bovine serum albumin for methyl-orange is destroyed by heat or dilute alkali.[152] However, the adsorption of methyl orange by denatured serum albumin is, at *p*H 6·8, lower than that by the native protein, although at *p*H 5·5, where the serum albumin is insoluble, the denatured form has the higher binding capacity. A similar effect is observed with $\beta$-lactoglobulin. Likewise soluble denatured egg albumin at *p*H 6·8 adsorbs methyl orange to a lesser extent than does the insoluble form at *p*H 5·5, the adsorption increasing with temperature. On the other hand, the adsorption of methyl orange and orange II on denatured precipitated lysozyme in the *p*H range 6·8 to 9·9 shows a marked increase at the higher values.[232] Egg albumin and serum albumin combine with congo red to form complexes; the number of dye molecules bound per protein molecule is roughly proportional to the concentration of the dye, but after complete heat denaturation this number increases by 4 for egg albumin and by 2 for serum albumin. Similarly, egg albumin exposed to temperatures of 65–70° combines with less congo red than does completely denatured egg albumin at 75°.[233] Bovine globulin shows no interaction with toluidine blue, methylene blue and rosirubine, whereas alkali-denatured $\gamma$-globulin binds these dyes. While the binding of bromphenol blue with serum albumin at *p*H 6·7 is lessened by alkali denaturation, such a denaturation of $\gamma$-globulin induces the binding of bromphenol blue.[244]

*X-ray diffraction.* When denaturation occurs in the solid state, x-ray studies may be carried out by comparing the diffraction patterns with those of well-known proteins of the $\alpha$ or $\beta$ keratin type.[29], [34], [37], [60], [74], [75] Before denaturation, proteins show the $\alpha$ pattern. In the



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initial heating (in the presence of water), partial crystallization of the protein occurs, as evidenced by the change in the diffraction pattern, diffuse rings becoming sharp and the number of rings increasing; application of high shear stress by extrusion or stretching gives rise to the $\beta$ pattern. Among the proteins which, by heat and mechanical working, give $\beta$-patterns are casein, $\beta$-lactoglobulin, haemoglobin, egg albumin, edestin, zein, peanut and soy bean proteins; the best patterns are obtained from $\beta$-lactoglobulin and egg albumin.[104], [105]

*Infra-red spectrum.* Infra-red spectroscopy gives important information on protein denaturation which is emphasized by the fact that the

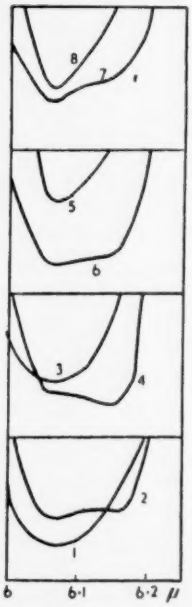


Fig. 1. Modification by denaturation of the infra-red spectrum of proteins in $D_2O$ solution.

(1) Egg albumin, *p*H 5·6; (2) egg albumin, *p*H 5·6, after 6 minutes at 100°C; (3) insulin, *p*H 4; (4) insulin, *p*H 4, after 6 minutes at 100°C; (5) insulin *p*H 10, after 6 minutes at 100°C; (6) the same solution, the *p*H having been altered to 7·8; (7) serum albumin, *p*H 4·6, after 20 minutes at 80°C; (8) the same solution, the *p*H having been altered to a value $> 10$. (After H. LENORMANT.)

absorption bands are the same in the dry state and in solution. On heat denaturation, for instance, an infra-red band appears at $6{\cdot}15\mu$, which can be more intense than the original band at $6{\cdot}05\mu$. Absorption in the $6{\cdot}90\mu$ region also increases (Fig. 1) and the intensity of the $6{\cdot}15\mu$ band increases with increasing time of heating. Now it is known that the $6{\cdot}05\mu$ band is characteristic of the $\alpha$ type and the $6{\cdot}15\mu$ band of the $\beta$ type of proteins.[192] It is important to note that the change of *p*H after heating does not change the spectrum; the displacement of the bands occurs at the same time as the heating and mainly just as the aggregation takes place. In alkaline medium (*p*H $>10$) the $\beta$ spectrum does not appear on heating, but, if after heating, the *p*H is shifted to the isoelectric point, coagulation of the protein occurs and the $6{\cdot}15\mu$

band appears.[243] Modification of the absorption spectrum may be obtained in certain cases by very mild treatment. For instance, the proteins of the glands of silkworms show the typical infra-red α-spectrum; however, by stretching or crushing of the concentrated solution, denaturation occurs, as shown by insolubility and appearance of β bands. Moreover, by dissolving the product in concentrated lithium bromide, the fibroin reverts after dialysis and the characteristic α spectrum is again observed.[198]

It is interesting, with respect to further discussion, to compare protein behaviour with that of the synthetic polypeptides. For instance, poly-DL-alanine (degree of polymerization about 500) can be readily dissolved in cold water. From this solution films can be cast, and the infra-red spectrum of such films shows an absorption band at 6·02μ. If the solution is heated, a gel separates out, which is not redissolved on cooling; the spectrum of the dried gel shows a distinct additional peak at 6·15μ. The dried gel is soluble in formic acid and the film cast from this solution shows only the 6·02μ peak, indicating that poly-DL-alanine has returned to its original condition. Moreover, the water soluble film can be converted into the insoluble form by rolling it out; the material so obtained shows the 6·15μ band of the β pattern and is orientated, as shown by measurements with polarized infra-red radiation.[226]

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*Ultra-violet spectrum.* After denaturation in alkali the absorption spectrum in ultra-violet light is frequently modified; but, as tyrosine shows a shift of maximum absorption from 282 to 278μ in going from *p*H 6 to *p*H 12, this effect is largely responsible for the variation of protein absorption.[238]

## Changes in the Size and Shape of the Particles and Aggregation due to Denaturation

*Molecular weight.* Most of the phenomena described above are closely related to changes in the shape or size of the suspended particles, such modifications being due to dissociation, swelling or aggregation of the molecules. Variations in the apparent molecular weight of denatured proteins have frequently been quoted, but with large discrepancies in the results of different authors. Such variations are probably due to differences in the experimental conditions, but also perhaps to diverse assumptions in the interpretation of the experimental data. Thus one finds in the literature that on heating serum albumin solutions polymerization occurs and that at 37°C the molecular weight reaches a maximum value of 4 times that at 20°C; at higher temperatures a dissociation process sets in which is superposed on the aggregation process.[100] At *p*H 3·6 the unheated and the heated serum albumin show a particle weight about twice that of the native isoelectric protein when measured

in solutions of 0·22 ionic strength.[102] On heating serum albumin at the limit of stability ($p$H 3·8) the sedimentation constant does not change but the solution ceases to be monodisperse when studied by light scattering.[24]

On heating egg albumin, the molecular weight increases rapidly within a few hours to a maximum and a sample which has reached this maximum value shows no further change when treated with urea.[230] It is generally considered that in urea denaturation the molecular weight does not change for egg albumin, serum albumin, serum globulin, gliadin, zein and pepsin.[47], [48], [88], [89] The molecules are split, however, in the cases of edestin, excelsin, amandin and haemoglobin,[47], [53] and it is found that egg albumin can also be split by sufficiently high concentrations of urea.[112] Likewise the denaturing effect produced by guanidine hydrochloride on pseudo globulin

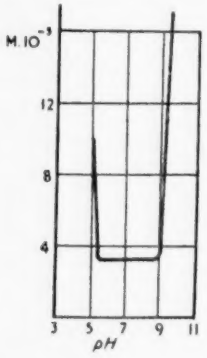


Fig. 2. Edestin—edestan transformation. $p$H dependence of the apparent molecular weight. (After M. L. Beeckmans and R. Lontie.)

depends on the concentration: 2 M guanidine hydrochloride causes an aggregation, the mean molecular weight being about twice that of the native protein; in 3 M solution the protein molecules split into halves, whereas a further concentration increase produces no additional change except that the solution becomes monodisperse.[89] Globulins of prunus seeds are stable in the range of $p$H 5·0–9·5; on the acid side denaturation gives rise to aggregation and on the alkaline side there is splitting of the molecules;[133] edestin is stable between $p$H 5·4 and 8·9, outside this $p$H range strong aggregation occurs giving rise to edestan[134] (Fig. 2).

*Particle shape.* The question of the change of shape of the protein molecules by denaturation has frequently been discussed. The large increase in viscosity and the decrease in diffusion constant, for instance, with serum albumin or pseudoglobulin in 8 M-urea or in guanidine hydrochloride,[63], [88], [89] are commonly interpreted in terms of the greater elongation of the denatured molecules. By using Polson's relationship to interpret the viscosity data on native, heat- and urea

denatured egg albumin, the following values of $\frac{a}{b}$,[70] at *p*H 8 in phosphate buffer of 0·02 M, are calculated: native 3·9, heat denatured 7·4, urea denatured 9·2; or, taking hydration into account,[81] $\frac{a}{b} = 1{\cdot}2$, 5·8 and 7·3 respectively. But, in such an interpretation of viscosities, the assumption has been made that the increase in viscosity is due to the stretching of the particles, which is completely arbitrary. Moreover, in numerous cases, such a postulated stretching is in contradiction to the data obtained by other techniques, including streaming birefringence or electron microscopy,[236], [239] while the part played by interaction forces or by solvation is neglected. An empirical attempt to take into account of solvatation has been made[224] by dividing the apparent coefficient of dissymmetry by $(1 + s)$,$^n$ where $s$ is the amount in $g$ of solvent taken up by 1 g protein, and $n$ a number between $\frac{1}{3}$ and 1; $\frac{a}{b}$ is so lowered by 42 per cent and 100 per cent respectively for human serum albumin and horse serum globulin in urea. A complete treatment of this problem, on a rigorous physical basis, has been developed by SHERAGA and MANDELKERN,[240] by introducing the concept of the effective hydrodynamic ellipsoid. By comparing the results of viscosity, diffusion and streaming birefringence, and by taking into account solvation and asymmetry, it was shown that urea denaturation causes swelling of the globular particles and not unfolding or stretching into an elongated particle. For instance, with horse serum albumin, according to the data of NEURATH and SAUM,[63] the radii of the particles have the values shown in the accompanying table.

| | | | | | | |
|---|---|---|---|---|---|---|
| $c_{\text{urea}}$ = | 0·5 M | 1·5 | 3·0 | 4·5 | 6·0 | 6·66 |
| $[\eta]$ = | 0·050 | 0·056 | 0·065 | 0·123 | 0·147 | 0·170 |
| $D \cdot 10^7$ = | 6·20 | 6·08 | 5·69 | 4·45 | 4·27 | 4·15 |
| $r$Å = | 38 | 39 | 41 | 51 | 54 | 56 |

Very recently OGSTON[238] has emphasized the difficulties in determining the dimensions of solute particles from dynamic properties of their solutions. He showed that precise values for the mass of the particles may be obtained but that values of dimensions can be at best only semi-quantitative.

*Aggregation.* It is generally recognized that aggregation plays an important part, at least as a secondary effect, in denaturation process[24], [79], [82], [102], [165], [188], [215], [220] and vice versa. That is to say, in most cases only denatured protein would be susceptible to aggregation[48], [102], [110], [122]. Sometimes aggregation seems to be a necessary step in denaturation, as shown by concentration effects. Thus in the pressure



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inactivation of trypsin or chymotrypsin, increase in concentration of the enzyme results in an increase in inactivation, while if the solution is sufficiently dilute, no significant amount of inactivation occurs.[187]

The reversible aggregation of insulin molecules is so well known that it needs no further comment here.[106], [126], [151], [218] Quantitative investigations were made, some years ago, on the aggregation of denatured egg albumin,[122], [137] and aggregates of 5 to 20 denatured molecules were found.[122] More recent and very exhaustive studies by streaming birefringence[208], [209], [213] of the heat denaturation of egg albumin have shown the relationship between protein concentration and particle size in glycerol solution at various *p*H values, ionic strengths and urea concentrations. The conditions leading to particles homogeneous in size and degree of aggregation were accurately determined, but unfortunately it is not certain that the glycerol does not affect the shape and the state of aggregation of the denatured particles. The length of the particles has been calculated assuming an axial ratio of about 50, though this may not be correct.[239], [240] By applying the theory of SMOLUCHOWSKI to the precipitation by $(NH_4)_2SO_4$ of heat denatured human serum albumin, it was deduced[175] that, after heating 10 minutes at 83°C and *p*H 6·8, only about one-half of the material is in the form of aggregates. These are composed of at least 5, 3 and 2 primary particles in heated solutions containing 1 per cent, 0·5 per cent and 0·25 per cent protein respectively. A systematic study of heat aggregation of horse serum albumin has recently been carried out;[166], [181], [184], [200] it was shown by several techniques that heat denaturation gives rise to elongated particles, the formation of which proceeds by end-to-end aggregation of the globular molecules. In such a linear aggregation, the length of the most frequent particles after heating for a time $t$ reaches a value $l$ which then remains practically constant for a considerable time. The values of $l$ and $t$ depend on concentration, *p*H, temperature and ionic strength (Fig. 3 and 4). After the period of stationary length, the particle size increases more or less regularly and, according to the experimental conditions, either coagulation or gelation of the solution may occur, since the variation with time of the particle polydispersity depends greatly on the temperature. A complete mathematical treatment of the formation and growth of the bead-like aggregates has been developed on the basis of polymerization kinetics,[239] the results being in good agreement with the experimental data. On the other hand, pressure denaturation of horse serum albumin gives rise to the formation of globular particles which seem to be very small droplets of concentrated protein solution. The most frequent diameter of these submicroscopic globules depends on concentration, *p*H, time of compression, temperature, pressure, ionic strength, etc.[239], [252] (Fig. 5).

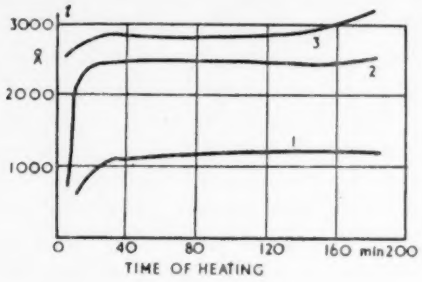


Fig. 3. Heat aggregation of horse serum-albumin. Variation of the length of the most frequent particles. $c = 2{\cdot}4$ per cent, $p$H $= 4{\cdot}15$, acetate buffer M/50. (1) $T = 57°$C; (2) $T = 80°$C; (3) $T = 100°$C. (After E. Barbu and M. Joly.)

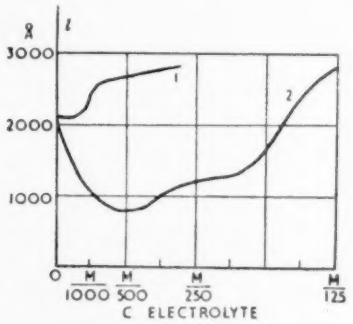


Fig. 4. Effect of electrolytes on heat aggregation of horse serum-albumin. Length of the aggregates. $c = 2{\cdot}4$ per cent, $p$H $= 4{\cdot}4$; 10 minutes heating at 80°C. (1) NaCl; (2) $BaCl_2$. (After E. Barbu and M. Joly.)

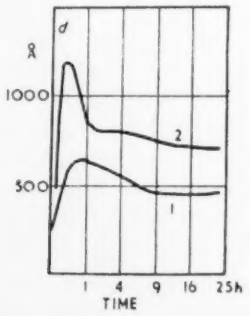


Fig. 5. Pressure denaturation of horse serum-albumin. Variation of the particle diameter with time of compression. $c = 6$ per cent, $p$H $= 7{\cdot}4$, $T = 37°$C. (1) $P = 6000$ kg/cm²; (2) $P = 1000$ kg/cm². (After E. Barbu, J. Basset and M. Joly.)

*Gelation.* Protein gelation appears to be closely related to denaturation,[88], [89] the process being more or less reversible. Thus, if fibrinogen in 30 per cent urea is completely denatured and then diluted with distilled water to 20 per cent urea, it gels after few minutes. If concentrated urea solution or solid urea is added to a gel formed in the presence of 10 or 20 per cent urea, the gel dissolves.[191] Moderate pressure gels serum globulin, but higher pressure liquefies the gel so obtained.[161] With all the proteins of horse serum, transparent gels can be obtained which liquify on heating but recover on cooling, although in a *p*H range more narrow than that for gelatine. In a narrow *p*H and concentration range one obtains successively reversible and irreversible gels by increasing the temperature, while the *p*H range of gelation enlarges with increasing concentration. According to the values of the concentration and *p*H, an increase in temperature leads either to gelation or to liquefaction; on cooling, the liquefaction is reversible but gelation is not. Acid gelation at room temperature is reversible whereas alkaline gelation does not reverse; isoelectric heat precipitation is not reversible without a large *p*H variation.[160]

## Particular Behaviour of Different Denaturing Agents and Inhibitors of Denaturation

The great diversity in behaviour on denaturation appears still more striking by comparing the effects of different denaturing agents on different proteins. Let us summarize only a few examples to show that there are several kinds of denaturation and that each type of denaturation seems not to be an "all-or-nothing" phenomenon, even on a molecular scale.

We have already seen that the behaviour of denatured serum-albumin is completely different, with respect to stability and aggregation, according as denaturation is due to heat or to pressure. Likewise heat treatment of human serum albumin is far less effective than urea denaturation when tested by papain digestion rates.[118] All proteins are affected by a sufficient concentration of H or OH ions; some proteins such as haemoglobin are denatured rapidly at *p*H values not far removed from the isoionic point; others appear to react reversibly with acid and base over a *p*H range extending from 1·5 to 11 or 12. Denaturation by acid or alkali is generally associated with the formation of a metaprotein, insoluble in the isoelectric region, but the chemical changes involved probably vary from protein to protein.[91] The part played by water in denaturation process is an important one. This is shown by the fact that the rate of denaturation of partially dried egg albumin is greatly decreased by reducing its water content.[30]

*Denaturation by enzymes.* Before enzymatic breakdown occurs, trypsin is able to exert a denaturing action on β-lactoglobulin similar

to that of urea, as characterized by volume effects. The same behaviour is observed with such systems as chymotrypsin-insulin or pepsin-$\beta$-lactoglobulin. Denaturation of aqueous solutions of $\beta$-lactoglobulin at $p$H 9·3, is accelerated by native trypsin but not by heat denatured trypsin.[173] Likewise the addition of trypsin to $\beta$-lactoglobulin in the presence of a small amount of urea has a pronounced catalytic effect on the rate of denaturation (Fig. 6), whereas trypsin has not any catalytic effect on the denaturation of serum albumin or egg albumin in the presence of 25 per cent urea at $p$H 9·25.[168] Similarly, thyroglobulin changes to the denatured form on heat treatment in the absence of enzyme[61] but active papain catalyzes this denaturation under conditions in which hydrolysis is negligible.[80] Egg albumin

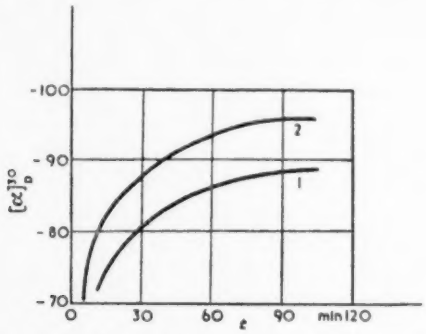


Fig. 6. Catalytic effect of trypsin on urea denaturation of $\beta$-lactoglobulin. 2·17 per cent $\beta$-lactoglobulin, 19 per cent urea, $p$H 8·25, ammonium-ammonia buffer M/10. (1) without trypsin; (2) 0·002 per cent trypsin. (After L. K. Christensen).

also, as shown by the biuret reaction, is denatured by pepsin at $p$H values at which enzymatic splitting is not detected.[155]

*Denaturation by chemical agents.* Diversity of behaviour is perhaps greater with chemical denaturing agents. For instance, we have seen how complex is the action of urea on fibrinogen, egg albumin, serum albumin or $\beta$-lactoglobulin. Moreover, the presence of oxygen doubles the rate of the urea denaturation of $\beta$-lactoglobulin;[246] in the case of egg albumin, the more the urea is diluted, the larger is the average size of the polydisperse particles. Lowering the temperature diminishes the size of the aggregates, as is shown by measurements in the ultracentrifuge, and also has the unexpected effect of increasing the rate at which the SH groups of the urea denatured protein react with ferricyanide.[29] Horse serum albumin, whether urea denatured or pressure denatured, behaves similarly with regard to streaming birefringence, while the aggregates formed by urea denaturation seem to be submicroscopic droplets of concentrated protein solution, the average

diameter varying between 200 and 800 Å.[248] In most cases guanidine hydrochloride shows denaturing effects analogous to those of urea, although lysozyme and pepsin are very resistent to guanidine denaturation, whereas Bence-Jones protein is very sensitive toward this reagent.[217]

Salt solutions sometimes promote denaturation, concentrated sodium salicylate in neutral solution denaturing haemoglobin[12] and legumin,[171] while serum globulin appears to be partly denatured by molar phosphate buffer or by ammonium sulphate on the acid side of the isoelectric point.[48] Likewise sodium tartrate, citrate, chloride or sulphate accelerate the biuret reaction of egg albumin, euglobulin and pseudoglobulin.[202]

Similar variety is observed with organic compounds. Thus the amount of acetone necessary to precipitate gelatin increases with increasing temperature, while the precipitability of egg albumin and of gelatin changes markedly with *p*H, whereas that of edestin much less.[92] The *p*H range within which precipitation of oxyhaemoglobin and egg albumin occurs is greater in the absence of formaldehyde than in its presence.[13] The rate of denaturation at 65°C of human serum globulin is increased by ethanol in proportion to its concentration from 0·05 to 0·6 M,[132] and, more generally, alcohols induce gel formation with serum proteins at room temperature and also promote heat gelation. Secondary liquefaction at high *p*H values is, however, prevented.[162] On the contrary no denaturation of the legumin of green peas or horse beans takes place in solution containing 2–30 per cent ethanol or propanol.[171]

Surface active agents frequently show a denaturing power as evidenced by the liberation of SH groups, increase of viscosity, changes in x-ray patterns, fibre formation, etc. The interactions of proteins with synthetic detergents have been extensively studied and the results have been reviewed.[141] Recent work on streaming birefringence induced in egg albumin solutions at room temperature by cationic agents like dodecyldimethylbenzyl, dodecyltrimethyl and dodecylammonium chloride or cetylpyridinium and cetyldimethyl-β-phenylethylammonium bromide, shows that it is difficult to describe in general terms the denaturation by cationic detergents.[249] In homologous compounds the chain length seems to play an important part in the denaturation process since although acetylamine accelerates the denaturation of egg albumin in proportion to the amount of amine present, decylamine is stronger in its action and dodecyl- and hexadecylamine cause even more rapid denaturation. Above a critical concentration, however, they have a stabilizing effect and prevent floculation.[219] With the anionic detergents precipitation is confined to the acid side of the isoelectric point although denaturation occurs

in both acid and alkaline regions. Relatively low concentrations of detergent exert a strong denaturing action compared with urea or guanidine hydrochloride. As an example of this, the intrinsic viscosity of serum albumin is increased from 0·043 to 0·22 by 8 M urea, but to 0·19 by 0·035 M sodium dodecylsulphate and to 0·25 by 0·17 M sodium dodecylsulphate.[103] All these effects are closely related to the ionic properties of lipo-protein association[177] as well as to the more general problem of the mutual solubility of colloidal polyelectrolytes.

*Inhibitors of denaturation.* Particular attention is to be paid to the more or less effective prevention of denaturation caused by a great number of substances. The significance of this in understanding the mechanism of denaturation we shall discuss below.

A great amount of work has been devoted to studying the inhibiting effect of fatty acids on the denaturation of serum albumin. With human serum albumin the stabilizing action increases with increasing chain length up to 12 C for low concentrations, although the maximum effect at higher concentrations is produced by the $C_7$ and $C_8$ acids.[120], [129], [130] In all cases the protein-fatty acid anion complex is of much greater solubility than the denatured protein itself.[110] Low concentrations of sodium caprylate, for instance, prevent the marked increase in viscosity of serum albumin which otherwise occurs in solutions of 6 M urea or 2·5 M guanidine hydrochloride. Instead there is a considerable decrease in the viscosity of the previously denatured protein (thus $[\eta] = 0{\cdot}044$ for serum albumin alone, 0·052 for serum albumin + 6 M urea + 0·02 M caprylate and 0·112 for serum albumin + 6 M urea). Ultrafiltration shows that in 0·005 M caprylate about 0·0024 M caprylate is bound by serum albumin, amounting to about 9 molecules of caprylate per molecule of serum albumin.[119] Even concentrations as low as 1 molecule of caprylate to each molecule of serum albumin result in marked stabilization.[129] Likewise the addition of low concentrations of sodium caprylate annuls the effect of heat treatment on the susceptibility of human serum albumin to papain digestion.[118]

Does the added substance combine with the protein in its native or in its denatured state? It seems well established, in the present case, that the fatty acids stabilize the native and not the denatured serum albumin, although experiments with other proteins and inhibitors show that this type of stabilization is by no means a general phenomenon.[129] Mandalate, for example, does not stabilize serum albumin in the native form, but it appears to act rather as a solubilizing agent for the partly denatured form, as deduced from papain digestion data.[148] One generally assumes that the binding of fatty acid anions by serum albumin is due both to electrostatic attraction by the positively charged guanidine and lysine residues and also to Van der Waals forces between the side chains of the anion and of leucine, isoleucine,



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valine and phenylalanine,[176] but further evidence on this is still required.[176]

Numerous other organic compounds have been studied from the standpoint of prevented denaturation. All the experiments lead to the conclusion that the requisite basic structure for stabilization is an anion with a non-polar group attached, the dissociation constant of the acid derived from the anion not being a primary factor. Addition of carbonyl or hydroxyl groups to the non polar group decreases the effectiveness of stabilization.[129] With a given criterion of stabilization, the relative efficacy of the various organic compounds can be determined. For example, with respect to the prevention of increase in

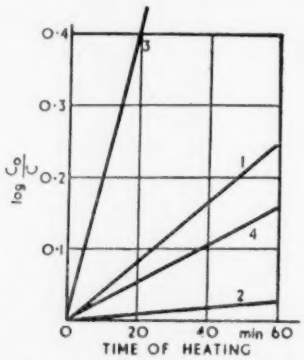


Fig. 7. Effect of $Mn^{++}$ on heat inactivation of lyzozyme. *p*H 7·9, 0·05 M borate buffer; $c_0$ = concentration of active lysozyme at zero time (= $14\gamma/cm^3$), $c$ = concentration of active lysozyme at time $t$. (1) $T = 70°C$, without $Mn^{++}$; (2) $T = 70°C$, $10^{-2}$ M $Mn^{++}$; (3) $T = 80°C$, without $Mn^{++}$; (4) $T = 80°C$, $10^{-2}$ M $Mn^{++}$. (After L. Gorini and F. Felix).

viscosity on adding 6 M urea to bovine serum albumin, the effectiveness decreases in the following order: dodecylsulphate, caprylate, 2-4-dichlorophenolate, picrate, phenylbutyrate, hexylmalonate, cinnamate and trichloroacetate.[145] A similar phenomenon is the formation of the C compound on heating bovine serum, which is inhibited by sugars like d-glucose, l-arabinose, sucrose, d-fructose, lactose, d-mannitol and d-xylose.[131] Further, cyanate stabilizes proteins against heat, heavy metal salts and alcohols.[167]

The different proteins show a wide diversity of behaviour with regard to denaturation inhibitors. For instance, the protective effect of sodium caprylate against the heat denaturation of egg albumin has been found to be unimportant,[212] whereas chicken egg albumin, as well as horse or bovine serum albumin, are almost completely protected when mixed with sodium thymonucleate.[186] In contrast to serum albumin, serum $\gamma$-globulin is not protected from urea denaturation by

caprylate,[130] and, instead of inhibiting, sodium caprylate accelerates the urea denaturation of fibrinogen.[191]

Certain mineral salts sometimes show inhibiting effects, an example being the increase in thermal stability of serum albumin with increasing NaCl content.[110] High concentrations of sodium sulphate seem to prevent urea denaturation.[119]

This last effect is perhaps related to the stabilizing properties of certain ions; the heat denaturation of trypsin at *p*H 7·9 is considerably retarded by $Ca^{++}$. Since all added compounds able to form a $Ca^{++}$ complex of low dissociation constant increase the rate of denaturation the evidence suggests that the stable form of trypsin is a complex calcium-trypsin. Likewise the percentage of heat-denatured bovine serum-albumin is lowered by $Ca^{++}$, the stable form of native serum albumin also being a complex calcium-serum albumin.[210] Similarly the ions $Mn^{++}$ and, to a less extent, $Co^{++}$ and $Mg^{++}$ combine with serum albumin at *p*H 7·9 to form complexes which are resistant to denaturation,[231] and an analogous effect is observed with lyzozyme and $Mn^{++}$, the latter decreasing the rate of heat inactivation and aggregation by forming a stable complex with lyzozyme[241] (Fig. 7).

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## Kinetics of Denaturation Processes

Before discussing the data on the reversibility of denaturation, we may find it useful to summarize the overall findings on denaturation kinetics. One of the most striking results is the high value of the heat of denaturation,[13], [31], [192] reaching for instance, 130,000 cal. in the case of the thermal denaturation of egg albumin, and its considerable dependence on the particular denaturing agent.[13], [32], [192]

The values and even the sign of the thermal coefficients of denaturation are still sometimes questionable. They are certainly very high and positive for heat denaturation.[2], [3], [4], [5] The case of urea denaturation is more complicated: with fibrinogen, the differential rate-temperature coefficient is positive[190] while with diphtheria antitoxin no negative temperature coefficient has been observed. However, the effect of a change in temperature is not uniform and may possibly tend to a minimum rate of inactivation at or below 0°.[150] With egg albumin, the temperature coefficient seems to be negative in 60 per cent urea,[17], [18] but in fact two reactions occur, denaturation and splitting, both having positive temperature coefficients, although as the urea concentration increases the second reaction is more affected by an increase in temperature than is the first. This explains the apparent negative coefficient.[112] Although β-lactoglobulin has a negative temperature coefficient at only 38 per cent,[144] it may be that for the first part of the denaturation the temperature coefficient is negative and very large, while for the later part of the process it is positive.

When $\beta$-lactoglobulin is denatured with sodium dodecylsulphate it shows a positive temperature coefficient.[207]

Similar difficulties arise in the determination of the reaction order, although in most cases denaturation seems, as a first approximation, to be a first order process, as, for instance, in the acid denaturation of oxyhemoglobin[13] and of egg albumin,[215] enzyme inactivation,[101] ricin denaturation,[189] alkaline denaturation of $\beta$-lactoglobulin[207] and serum albumin,[9], [10] and the heat denaturation of lyzozyme,[241] egg albumin[233] and $\beta$-lactoglobulin.[234] On the other hand alkaline denaturation of egg albumin follows approximately a bimolecular course.[91] In the inactivation of trypsin however, the reaction is monomolecular or bimolecular according to the $p$H range considered.[33] The rate of heat denaturation of human serum globulin decreases progressively after about 40 per cent of the protein initially in solution has been precipitated[138] and similar behaviour is observed in the urea denaturation of diphtheria antitoxin[150] and fibrinogen[190] and the alkaline denaturation of serum albumin,[250] the first order primary reaction being followed by changes characterized by a gradual decrease in reaction rate.

The EYRING theory of absolute rate processes can be well applied to protein denaturation.[50], [65], [78] It leads to the equations:

$$k' = \frac{kT}{h} e^{\frac{-\Delta F_+^\ddagger}{RT}} = \frac{kT}{h} e^{\frac{-\Delta H_+^\ddagger}{RT}} e^{\frac{\Delta S_+^\ddagger}{R}} = \frac{kT}{h} K_+^+ \frac{\gamma_n}{\gamma_+^+}$$

where $k'$ is the specific rate constant in sec.$^{-1}$, $\Delta F_+^+$, $\Delta H_+^+$ and $\Delta S_+^+$ are respectively the increases in free energy, enthalpy and entropy due to the formation of the activated complex, $K_+^+$ is the equilibrium constant and $\gamma_+^+$ is the activity coefficient of the activated complex. $\gamma_n$ represents the activity coefficients of reagents, $T$ is the absolute temperature, $h$ is PLANCK'S constant, $k$ is BOLTZMANN'S constant and $R$ is the gas constant. The free energy of activation is therefore given by

$$\Delta F_+^+ = RT \ln \frac{kT}{hk'}$$

the activation enthalpy is given by

$$\Delta H_+^+ = RT^2 \frac{d \ln k'}{dT} - RT$$

and the activation entropy is calculated from

$$\Delta S_+^+ = R\left(\ln \frac{kT}{hk'} + T \frac{d \ln k'}{dT} - 1\right)$$

The volume increase due to the formation of the activated complex is given by

$$\Delta V_{+}^{+} = - RT\left(\frac{d \ln k'}{dP}\right)_{T} = - RT\left(\frac{d \ln K_{+}^{+}}{dP}\right)_{T}$$

$P$ being the hydrostatic pressure. The following table shows a few values of these thermodynamic quantities:[65], [169]

| | $p$H | $t$°C | $k'$ sec$^{-1}$ × $10^4$ | $\Delta H_{+}^{+}$ | $\Delta S_{+}^{+}$ |
|---|---|---|---|---|---|
| Insulin | 1·5 | 80 | 1 | 35,600 | 23·8 |
| Pepsin | 6·44 | 25 | 5·47 | 55,600 | 113·3 |
| Trypsin | 6·5 | 50 | 0·283 | 40,160 | 44·7 |
| Egg albumin | 5·0 | 65 | 2·54 | 132,000 | 315·7 |
| ,, | 5·9 | 65 | 2·5 | 135,000 | 325 |
| ,, + urea 0·6 g/cm$^3$ | 5·9 | 23 | 3 | − 3000 | − 85 |
| Haemoglobin | 5·7 | 60·5 | 4·3 | 75,600 | 152·7 |
| Oxyhaemoglobin | 6·5 | 60 | 2·3 | 76,500 | 1540 |
| ,, + 30 per cent alcohol | 6·5 | 33 | 6·8 | 120,100 | 319 |
| ,, + 0·001 M$(NH_4)_2SO_4$ | 6·76 | 64 | 3400 | 76,500 | 153 |
| ,, + 3·03 M $(NH_4)_2SO_4$ | 6·76 | 64 | 0·24 | 119,800 | 276 |

In general, protein denaturation involves a large $\Delta V_{+}^{+}$, for instance, it is 100 cm$^3$ for globulin inactivation[132] and 40 cm$^3$ for the denaturation of staphylococcic antitoxin in the first third of the process, although less than 22 cm$^3$ after 80 per cent reaction.[128]

The large values of the energy and entropy of activation in protein denaturation are illusory since the customary method of calculation by comparison of rates keeping only the $p$H constant includes, in addition to the true energy of activation, the heat of dissociation of all ionic species involved in preparing the molecules for activation.[51] In this way we see that the apparent heat of activation is not at all representative of the true denaturation reaction, but signifies only a composite value for many reactions, of which only one is the actual denaturation. This is why the apparent heat of activation can vary over such a wide range.[216]

The primary reactions most generally assumed are proton dissociations, in consequence of the variation of denaturation rate with $p$H. Thus the rate of alkaline denaturation of $\beta$-lactoglobulin is inversely proportional to the 1·1 power of the hydrogen ion concentration,[207] while the plot of the logarithm of the reaction rate, during the denaturation of egg albumin, versus $p$H gives a straight line of slope − 1.[211] The rate of alkaline denaturation in the latter example is proportional to $[OH^-]^1$.[91] In the urea denaturation of fibrinogen the reaction rate is increased by lowering the $p$H below 7, whereas between $p$H 7 and 8·6 it is nearly constant.[190]



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For ricin denaturation, all the experimental data can be explained by assuming that a single proton dissociation leads to the ionic form which undergoes denaturation.[189] In the case of pepsin, the $p$H effect shows that the reactive form differs by 5 protons from the stable form,[49], [51], [53] and the following thermodynamic quantities are found for the ionization processes:[65] for $P_0 \rightarrow P_{5+}^{+}$, $\Delta H_{+}^{\pm} = 67{,}670$, $\Delta F_{+}^{\pm} = 26{,}955$, $\Delta S_{+}^{\pm} = 135{\cdot}9$; for $P_5 \rightarrow P_{5+}^{+}$, $\Delta H_{+}^{\pm} = 22{,}360$, $\Delta F_{+}^{\pm} = 19{,}737$, $\Delta S_{+}^{\pm} = 8{\cdot}8$; and for $P^0 \rightarrow P^5$, $\Delta H = 45{,}110$, $\Delta F = 7218$ and $\Delta S = 127{\cdot}1$. Likewise, in the denaturation of egg albumin, the reaction rate varies at low acidities according to the equation $k = k'[\mathrm{H}^+]$. At high acidities we find $k = k'[H^+] + k''[H^+]^2$, and by assuming that denaturation involves the disappearance of 1 or 2 proton dissociations (depending on the $p$H), one can write,[215] as a picture of the process,

$$P \underset{(K_1)}{\overset{H^+}{\rightleftharpoons}} P^{+1} \underset{(K_2)}{\overset{H^+}{\rightleftharpoons}} P^{+2}$$
$$\downarrow (k_1) \qquad \downarrow (k_2)$$
$$D \qquad D$$

Here $k' = \dfrac{k_1}{K_1}$ and $k'' = \dfrac{k_2}{K_1 K_2}$; while $k'$ and $k''$ can be determined from experimental data (for instance, at 25°C $k' = 3{\cdot}11 \times 10^{-4}$ and $k'' = 7{\cdot}91 \times 10^{-3}$, while at 39·6°C $k' = 5{\cdot}88 \times 10^{-3}$ and $k'' = 4{\cdot}27 \times 10^{-1}$), $K_1$, $K_2$, $k_1$, $k_2$, and therefore the true denaturation velocities, are undetermined.[215]

### Data Suggesting Reversal of Denaturation

Rather numerous experimental findings have suggested that protein denaturation may be a reversible process. In several cases it seems that reversible and irreversible denaturation occur either successively or simultaneously, the denaturation thus appearing to be partly reversible. However, owing to the extremely diverse properties shown by protein molecules, it is always difficult to ascertain that the native and reversed proteins are exactly identical. This is all the more so as different samples of the same native protein sometimes show different behaviour (for instance, horse serum albumin with respect the pressure effect).[252] Thus the denaturation of a given protein by a given denaturing agent may or may not appear to be reversible according to the chosen criterion of denaturation. This explains the frequent discrepancies between the conclusions of different authors. In most cases denaturation is reversible only with regard to certain properties; nevertheless in a few cases the reversibility seems complete. For instance, the crystalline product from reverted fibrils of insulin is not significantly different from native insulin in its crystallization (in presence of zinc), biological activity, ultracentrifuge pattern or fibril formation. Tests

for changes in labile groups, such as amino and disulphide, are also negative.[151] Similarly, renatured chymotrypsinogen is identical with native chymotrypsinogen with respect to crystallizability, solubility, activation to δ-chymotrypsin, sedimentation rate and heat behaviour.[204] Reversed urea-denatured β-lactoglobulin does not differ from the native protein in its crystallization, optical activity, titration of SH groups, dilatometric or viscosity behaviour or rate of trypsin digestion.[225] But frequently the reversal of denaturation is only apparent and not quantitative; the properties of the native and reversed proteins are only approximately the same, but not exactly. For instance, methaemoglobin prepared from coagulated haemoglobin by reversal of coagulation has the same solubility within 2 per cent as normal methaemoglobin.[20] And more precisely, the solubility curves in $(NH_4)_2\ SO_4$ of native and reversed bovine methaemoglobin are

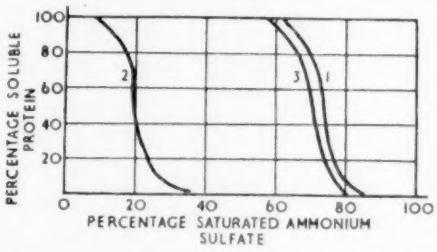


Fig. 8. Reversal of salicylate denaturation of bovine methaemoglobin. Solubility curves. $p$H = 6·06, $T$ = 18°C. (1) native; (2) denatured; (3) reversed. (After J. ROCHE.)

practically the same except that a little excess precipitation occurs in the reversed protein in 65 per cent ammonium sulphate[52] (Fig. 8). Likewise the reversed urea-denatured horse pseudo-globulin resembles, but is not identical with, the native protein in respect to molecular size, shape and electrophoretic properties. Although electrophoretically homogeneous, it shows a higher mobility above, and a lower mobility below, the isoelectric point than does the native protein.[87], [89] We shall now discuss the most important data on the reversal of denaturation.

*Reversal by pH changes.* The more soluble is a denatured protein, the more easily can its denaturation be reversed.[23] This, however, does not mean that solubility is the only factor which determines the extent to which reversal takes place or indeed whether it can take place.

The reversal of the denaturation of serum albumin was presumably observed by MICHAELIS and RONA,[1] but the first systematic study seems to be due to SPIEGEL-ADOLF,[8] who established that heat coagulated serum albumin can be dissolved in alkali, dialysis of the product giving a water soluble protein which is also soluble in salt

solution and shows the same conductivity, optical rotation, heat behaviour and immunochemical properties as the native protein. Likewise, it is possible to prepare a crystalline, soluble, heat coagulable serum albumin on neutralization of the acid denatured protein. However, it is still not clear why any reversal should result from neutralization, since native proteins are frequently unstable at their isoelectric points. Moreover, only 75 per cent of the denatured serum albumin is converted into the soluble protein. When soluble serum albumin is produced by the reversal of denaturation, there is a corresponding disappearance of the SH groups.[23] Native horse serum albumin and that recovered after denaturation by acid in acetone show different solubilities in increasing concentrations of $(NH_4)_2 SO_4$, (at *p*H 6·16 and 22°C), the molecular weights being the same[69] (Fig. 9).

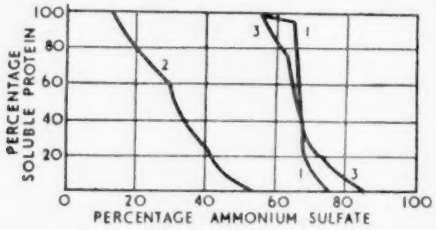


Fig. 9. Reversal of acid acetone denaturation of horse serum-albumin. Solubility curves. *p*H 6·1, $T = 22°C$. (1) native; (2) denatured; (3) reversed. (After J. Roche and M. S. Chonaiech.)

Insulin can be completely inactivated by treatment with ethanol and HCl, or by mixtures of other strong acids with primary or secondary alcohols, although complete recovery of potency and of the original precipitation point can be obtained by treatment with dilute NaOH.[14] The reversion of insulin fibrils is optimal for 2 per cent insulin in 0·03 M alkali at 0°C and with 45 minutes treatment.[151]

The denaturation of globin prepared from haemoglobin by the acid in acetone method is reversible, the globin obtained by reversal being soluble even if the globin is first precipitated by trichloracetic acid or heated to 100°C.[21] Moreover globin and haem obtained by splitting horse haemoglobin with acid can be recoupled to form a haemoglobin with the same molecular weight and the same *p*H stability characteristics as native haemoglobin, although electrophoretic experiments lend doubt as to the identity of the native and the recoupled haemoglobin.[67] Moreover, acid denaturation of haemoglobin is only partially reversible and if acidified haemoglobin is rapidly neutralized, all the protein is precipitated. But if it is first made slightly alkaline and then, after a few seconds, is brought to neutrality, only a third of the protein is precipitated, the soluble two-thirds having regained the

properties of native haemoglobin.[32] Likewise pepsin which has been completely denatured and inactivated in a solution of *p*H 10·5 recovers some of its activity when allowed to stand at about *p*H 5·4 for 24 to 48 hours at 22°C. The protein then shows the same solubility, crystalline form and specific proteolytic activity as does the original crystalline pepsin.[22] On the other hand, as judged by the optical rotation, solubility and crystallizability, no reversal of denaturation can be obtained by allowing *p*H-denatured β-lactoglobulin to stand in dilute acid or alkali, followed by dialysis at the isoelectric point.[207] As we have seen, the transformation edestin—edestan occurs outside the *p*H range 5·4–8·9, lower the edestan average molecular weight, higher acidity and lower the ionic strength; the transformation is reversible at low *p*H, and it is irreversible at high *p*H.[139])

*Reversal by dilution or dialysis.* Denaturation of haemoglobin by salicylate is almost completely reversed when the salicylate is removed by dialysis or even when the solution is diluted with water.[32] In the case of serum albumin denatured by urea, the reversal is only apparent and is not quantitative. Indeed, for the optical rotation, no difference is observed within the experimental accuracy between the rotations ($[\alpha_D]$ is about $-61°$) of native and of regenerated serum albumin.[146] Removal of urea from solutions of urea-denatured human serum albumin lowers the papain digestion rate nearly to the original level.[118] When an insoluble protein regains its solubility, SS groups, which are detectable in the denatured protein, disappear; there is no evidence that SS groups appear before the protein loses its solubility.[42] Reversibility of urea denaturation is also indicated by the biuret reaction.[170] More precisely, upon removal of the urea by dialysis, serum albumin separates into two fractions differing from one another in solubility. The more soluble fraction is apparently reversibly denatured protein, whereas the more insoluble fraction is irreversibly denatured protein, the amount of which increases with the urea (or guanidine hydrochloride) concentration causing denaturation, and reaches a maximum of 15 per cent in 6 M urea (or 2 M guanidine hydrochloride). The protein obtained after fractional precipitation of the more soluble fraction has about the same molecular weight, the same apparent molecular size and shape and the same antigenic specificity as has the native protein, but it differs from it in its solubility in $Na_2SO_4$, in its crystallizability, its electrophoretic mobility and in its antigenic activity.[88], [89], [90] Similarly, pseudoglobulin, on removal of the denaturing agent by dialysis separates into two fractions considered to be reversibly and irreversibly denatured. The ratio of these fractions depends on the concentration of the denaturing agent, guanidine hydrochloride leaving a larger fraction irreversibly denatured than does urea.[89] The behaviour of egg albumin is quite different, and



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this protein does not revert to a state in which it is soluble at the isoelectric point. Indeed, the protein obtained after removal of the urea (as well as after cooling of the heated solutions) has a considerably larger average particle weight than has the original protein,[86] while its relative viscosity remains fairly high and its antigenic specificity differs from that of native egg albumin.[11] Similarly, denaturation of serum albumin, $\gamma$-globulin, pepsin, chymotrypsinogen or lyzozyme by KSCN or guanidine hydrochloride is an irreversible process; the original properties of the proteins may be only partially restored or may even be considerably altered if the product is freed from the denaturing agent.[221] On the other hand, with $\beta$-lactoglobulin, removing the urea (or guanidine hydrochloride) by dialysis, leaves a protein which in the crystalline form has all the characteristics of native $\beta$-lactoglobulin.[207]

As we have already seen, trypsin is stabilized in its active form by calcium ions and the complex calcium-trypsin is reversibly dissociable, active trypsin transforming itself into the inactive form after calcium release. The life time of this form is short, however, and the protein is more or less rapidly transformed into an irreversibly inactive form. Likewise serum albumin is stabilized in its active form by calcium ions, the corresponding calcium-serum albumin complex being reversibly dissociable, although the protein obtained after calcium release shows a tendency to be irreversibly denatured.[210] In these cases, therefore, the possibility of reversal of denaturation is closely related to the presence of calcium ions, while being, in addition, restricted to a short time interval before the irreversible denaturation occurs.

*Reversal by cooling or heating.* Reversal of heat denaturation by cooling has been found to take place with serum albumin, globin, pepsin and chymotrypsin, but in none of these cases is the reversal complete.[31], [101] With trypsin, only in acid solution can reversal of heat denaturation by cooling be effected without irreversible inactivation; in alkaline solutions (in which, however, the temperature of half denaturation is low) there is irreversible inactivation. If trypsin is heated to 60°C in 0·05 M acid solution, it is converted into a protein completely precipitated by quarter saturated $(NH_4)_2SO_4$, but when the heated trypsin is cooled, it changes back into the original form which is not precipitated by quarter saturated $(NH_4)_2\,SO_4$. Moreover, if trypsin is heated or cooled to about 40°C, a definite fraction is precipitated by salt,[31] indicating, as mentioned below, the existence of an equilibrium. The loss of activity on heating is very rapid and is completely reversible for short times of heating only. If, after losing its activity at high temperatures (or in alkaline solutions), trypsin is allowed to stand, the loss in activity gradually becomes irreversible.[33], [101]

Crystalline soya bean trypsin inhibitor protein undergoes denaturation on heating which is reversed on cooling, and no distinct difference can be experimentally detected between the originally native and the reversed protein.[159] Likewise, within a restricted range of *p*H's and

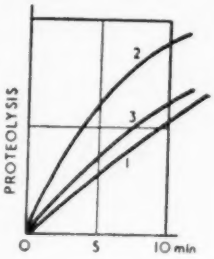


Fig. 10. Reversal by cooling of heat denaturation of β-lactoglobulin. 2 per cent lactoglobulin, 10 per cent trypsin, $T = 30°C$, *p*H 7. (1) native; (2) heat denatured (1 minute at 100°C); (3) reversed (20 hours later). (After K. Linderström-Lang and R. D. Hotchkiss.)

protein concentrations, crystalline chymotrypsinogen undergoes thermal denaturation which is wholly reversed upon cooling.[204] The heat denaturation of β-lactoglobulin can also be reversed by cooling, as is shown by trypsin digestion studies[55] (Fig. 10).

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The reversal by heating of the urea denaturation of β-lactoglobulin is an interesting phenomenon. This reversal is due to the large

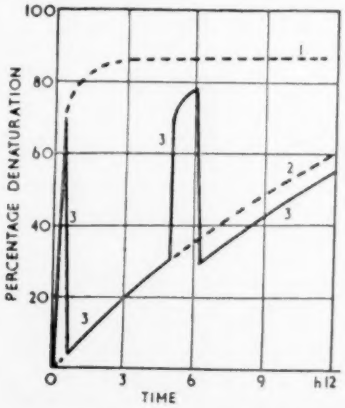


Fig. 11. Reversal by cooling of urea denaturation of β-lactoglobulin. 2 per cent β-lactoglobulin, 38 per cent urea, 0·1 M NaCl, *p*H 5·17. (1) $T = 0°C$; (2) $T = 37·4°C$; (3) 6 minutes at 0°C; 5 hours at 37·4°C; 1 hour at 0°C and 6 hours at 37·4°C. (After C. F. Jacobsen and L. K. Christensen.)

negative temperature coefficient of the urea denaturation of β-lactoglobulin (see above); denaturation is very rapid at 0°C but is nearly completely reversed if the temperature is increased (Fig. 11). The reversed protein is identical with the starting material with respect to its crystal habit.[144]

*Reversal of denaturation by pressure.* Partial reversal of heat denaturation is often effected by high pressure treatment, although as we have seen above, pressure is itself a denaturing agent. However, as we have emphasized several times, denaturation is completely different on heating and on compression, principally in regard to secondary effects like aggregation or particle dissymmetry. Further, the antagonistic actions of alternating heat and pressure treatments have been demonstrated.[239] Thus the tryptic digestion of casein at high temperatures is accelerated by compression although a relatively small volume change is involved. This probably means there is a preferential repression of the denaturation of the enzyme[206] in spite of the weak

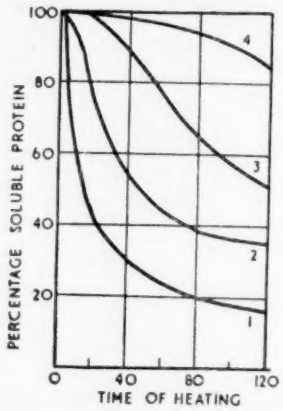


Fig. 12. Pressure reversal of heat denaturation of human serum globulin. Temperature of heating 65°C, *p*H 6·0, phosphate buffer. (1) atmospheric pressure, 0·3 M ethanol; (2) atmospheric pressure, without alcohol; (3) 700 atm., 0·3 M ethanol; (4) 700 atm., without alcohol. (After F. H. Johnson and D. H. Campbell.)

effect of pressure on trypsin at low *p*H.[187] Likewise the rate of heat denaturation of human globulin is reduced by hydrostatic pressure up to 400 kg/cm², while pressure of 750 kg/cm² greatly retards the rate of precipitation and causes a marked initial time lag of nearly one hour, during which only slight precipitation occurs.[114] Pressure retards the denaturation, even in the presence of ethanol, approximately independently of concentration up to 0·3 M[138] (Fig. 12). Similar results are found with invertase[135] and with luciferase.[94], [95]

In the pressure reversal of denaturation, the reversed protein is often different from the native. Thus, when conalbumin is denatured by heating at 62°C for 8 minutes and is then kept at 2000 kg/cm² for 20 hours, the properties of the partially regenerated protein approach those of the initial material. However, this renatured protein differs both from native and from heat-denatured conalbumin. Similarly,

heat-precipitated egg albumin can be brought into solution by high pressures and is also regenerated under pressure in the presence of glucose. Although pressure can completely restore the solubility of both proteins, certain other properties are not fully regenerated.[222] Moreover, the reversal effect of compression depends largely on the actual pressure. For instance, with egg albumin in water or in phosphate buffer at *p*H 6, the denaturation at 61°C is counteracted by pressure as long as it remains below 3000 kg/cm². Under 6000 kg/cm² to 7000 kg/cm² the denaturation becomes more rapid than at atmospheric pressure.[172] Similarly, in the case of heated human serum albumin at *p*H 6 in phosphate buffer, the equilibrium is shifted towards the native form by pressures greater than 1000 kg/cm², although not too high. The initial pressure necessary for denaturation increases with increasing temperature,[192] and after heating for 15 minutes at 70°C, practically all the precipitated protein goes into solution in 20 hours, under a pressure of 2000 kg/cm². (In contrast with egg albumin, serum albumin can be renatured under pressure even though it has stood for a considerable time in the denatured state.) At a pressure of 1000 kg/cm² the reaction does not proceed to completion even after 70 hours, while a pressure of 3000 kg/cm² causes the denatured protein to dissolve rapidly initially. However, after prolonged action at this pressure denaturation begins again. The viscosity and the optical rotation of the initial and of the pressure treated proteins are identical, although the denatured sample possesses a higher viscosity and a greater rotation. Tryptic digestion also proceeds at the same rate for the native and renatured protein, in contrast to the more rapid digestion rate for denatured serum albumin. Thus, when the denatured protein is subjected to high pressures, all the factors seem to change in a direction opposite to that during heat denaturation.[185]

Pressure treatment can also bring about reversal of denaturation by a chemical agent, e.g. the reversal of the denaturation of enzymes by narcotics. This has been shown in the study of the effect of urethane on invertase activity[140] and in the experiments on luminescence inhibitors.[84], [85], [97], [98], [108], [116], [136], [156], [214] On the other hand, pressure has little or no influence on the denaturation of methaemoglobin by methane or by salicylate.[178]

*Reversal by chemical treatment.* Organic compounds, which behave as inhibitors of denaturation can, in certain cases, reverse denaturation more or less completely. Thus, the addition of sodium caprylate to serum albumin solutions previously denatured by 6 M urea results in a prompt and pronounced decrease in viscosity as well as in a lowering of the papain digestion rate, the effects decreasing with the time of exposure to urea.[119] Similarly, reversal of denaturation of bovine serum albumin by salicylate, trichloracetic acid or heat may be effected



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by the addition of KCN,[12], [15], [32] although this reversal is limited to 90–95 per cent for denaturation by salicylate or trichloracetic acid, and to only 25 per cent for heat denaturation.[52] The method generally used for resolubilizing coagulated silk are related to this renaturation process: in one method refined cocoons are dissolved (in 10 to 20 minutes) by a very concentrated cuprammonium complex salt solution prepared by dissolving $Cu(OH)_2$ in a concentrated aqueous solution of $NH_3$. Following complete solution of the cocoons, the solution is diluted several times and is then neutralized by dilute HCl with very gentle stirring. A second method consists in boiling the silk thread in a concentrated solution of $CaCl_2$ and a copper salt, with subsequent dilution; the fibroin solutions so obtained show the same behaviour as the liquid silk from silk worms.[157]

### Remarks on Complete, Incomplete and Apparent Reversals of Denaturation

As emphasized by Kunitz[159] and as we have noted above, it is practically impossible to decide categorically whether a reversal of denaturation can be complete. To do this it would be necessary to prove that every physical, chemical and biological property of the native protein is restablished by reversal of denaturation. Nevertheless, reversibility can be studied with respect to a given property and, in certain cases, equilibrium between native and denatured protein can be reliably observed. This is true at least for some definite step in the denaturation process, and it seems that the urea denaturation of $\beta$-lactoglobulin proceeds by simultaneous or consecutive reactions, at least one of which proceeds to a temperature dependent equilibrium between the denatured and the native protein.[144]

*Equilibrium: native* $\rightleftarrows$ *denatured protein.* In several cases the existence of such a mobile equilibrium has been demonstrated on the basis of some given criterion of denaturation, although sometimes the conditions of reversibility are satisfied only over a very narrow range of *p*H, concentration, temperature and time of treatment (see later). Moreover, when there is a mobile equilibrium between the native and denatured forms, denaturation can be caused by the addition of any substance which has a greater affinity for the denatured than for the native form.[32] Let us now summarize a few examples of such well established equilibria.

In the range of temperature of 35 to 50°C a solution of soybean trypsin inhibitor consists of a mixture of the native and denatured forms in equilibrium with each other. Although this equilibrium is slowly established, its final value at any temperature is the same whether a heated denatured solution is cooled or whether a fresh solution is heated to that temperature[159] (Fig. 13). The corresponding

thermodynamic data are as follows: $\Delta H = 57{,}000$, $\Delta S = 180$, $\Delta H_{1+}^{+}$ for denaturation $= 55{,}000$, $\Delta H_{2+}^{+}$ for the reversal of denaturation $= -1900$, $\Delta S_{1+}^{+} = 95$, $\Delta S_{2+}^{+} = -84$. Salicylate, in solutions not concentrated enough to denature haemoglobin completely, produces an equilibrium mixture of native and denatured protein, with higher salicylate concentrations producing a higher percentage of denaturation. At any given salicylate concentration, the percentage denaturation is the same whether one starts with the native or with denatured haemoglobin.[32] Likewise, under conditions precluding irreversible inactivation, a definite fraction of trypsin is in the active native state and a definite fraction is in the inactive denatured form. The percentage

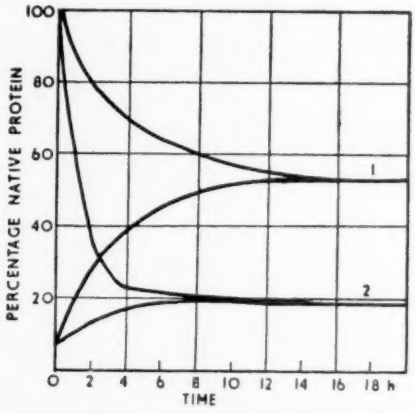


Fig. 13. Reversible heat denaturation of crystalline soybean trypsin inhibitor. *p*H 3·0. (1) $T = 45°C$; (2) $T = 50°C$. (After M. KUNITZ.)

inactivation at a given temperature is the same whether the solution is heated or cooled to that temperature (for instance, in 0·01 N HCl trypsin is almost completely native at 40°C and almost completely denatured at 50°C). More generally, the percentage denaturation at equilibrium depends only on the conditions at equilibrium and is therefore the same whether one starts with the native or denatured protein, so that by heating trypsin at any *p*H, a temperature may be reached at which the enzyme is half denatured. This temperature is very sensitive to the *p*H, and is lowered by either acid or alkali since ionization of the trypsin causes the equilibrium between the native and denatured enzyme to be shifted in favour of the denatured form. Denaturing agents also bring about such a shift. The concentration can vary by as much as ten times without any effect on the percentage of denaturation, so that the kinetics of denaturation must therefore be the same as the kinetics of the reversal of denaturation. In fact,

$\Delta H = 67{,}000$, $\Delta F = 40{,}160$, and the heat of regeneration of active trypsin is $-27{,}440$ while the corresponding loss of entropy is $-168{\cdot}4$.[16], [31], [33]

Sometimes several successive equilibria are involved, as in the formation of a urea-protein complex which has been isolated, by the methyl orange spectrum method, from the subsequent denaturation products.[152] In most cases the different successive or simultaneous equilibria invoked to explain the experimental data are only more or less arbitrary assumptions. Thus, in the case of $\beta$-lactoglobulin, it has been assumed, in interpreting the heat reversal of urea denaturation, that the native protein exists in two equilibrium forms, the rate of transformation being high, while the predominating form at low temperature is characterized by its susceptibility to urea denaturation. In this way denaturation is postulated as proceeding in two steps, one of which involves the equilibrium ratio between the two forms of $\beta$-lactoglobulin.[173] The existence of several forms in equilibrium is also assumed in interpreting the behaviour under pressure of trypsin or chrymotrypsin (see above). This assumption explains the fact that these enzymes are most active near $p$H 8 and that they show little or no activity at low $p$H values. It is also easy to assume an equilibrium between two forms, active and inactive, the latter being much more resistant to pressure. The increase in inactivation with increasing concentration, suggests that, under the influence of pressure, an irreversibly inactivated enzyme is formed by the reaction of two or more molecules of enzyme, probably in the active form. In view of the time effect, it seems that a portion of the active form is converted by pressure into the irreversibly inactive form, while the remainder is very resistant to inactivation. The latter part may already exist as the reversibly inactive form or may be converted into this form by the high pressure, but when the pressure is released, the inactive form would be expected to revert to the equilibrium amounts of the reversibly inactive and active forms, leaving the irreversibly inactivated enzyme unchanged. By applying pressure a second time, a portion of the enzyme is converted to the irreversibly inactivated form while the remainder goes to the reversibly inactive form, so that repeated application and release of pressure will lead to complete conversion of the enzyme to the irreversibly inactive form.[187] All these processes are summarized in the diagram:

$$P \downarrow \begin{matrix} E_a \\ E_i \end{matrix} \overset{P(H)^+}{\underset{(O)H^-}{\rightleftharpoons}} E_r$$

where $E_a$ refers to the active form, $E_r$ to the reversibly inactive form, and $E_i$ to the irreversibly inactive enzyme. At present, however, no

direct experimental proof has been given of the operation of such a mechanism.

As we have seen above, an equilibrium exists between native and reversibly heat-denatured chymotrypsinogen, but to avoid irreversible denaturation, it is necessary to limit the conditions to a rather narrow range of *p*H, concentration and temperature. Within the appropriate *p*H range (2–3) the amount of denatured chymotrypsinogen is a function of the third power of the hydrogen ion activity, while both denaturation and its reversal are first order reactions with respect to protein. It has been suggested that reversible heat denaturation of chymotrypsinogen consists largely in a partial dehydration of a protein molecule.[204] Indeed, the very high reaction rate suggests a reaction involving small molecules, while the behaviour of solutions of reversibly denatured chymotrypsinogen towards dilute salt solutions is parallel to that of lyophobic sols. Thus the heat of hydration is large enough to cause a marked decrease in the interaction between protein and water, and hence in protein solubility, by heating to even moderate temperatures, while the large entropy change in reversible denaturation is in accord with the view that the water of hydration is similar in structure to ice. More precisely, the heat of adsorption of the first layer of water between adjacent hydrophilic surfaces in dry proteins lies between $-1060$ and $-3800$ cal. per mole of water adsorbed,[107] while the lower limit to the heat of adsorption of water by dry proteins in the absence of air is about 6000 cal./mol.[179] Although the lattice energy of chymotrypsinogen is probably large, the heat of hydration is sufficiently great for the protein to have a negative heat of solution: at *p*H 3 (in 0·5 and 0·75 saturated $(NH_4)_2\,SO_4$) the heat of solution of chymotrypsinogen is about $-14{,}000$ while at *p*H 5 in salt-free solution it is $-8800$ cal. ([138,204]). If the heat of fusion of ice is taken to be 1440 cal./mol., about 69 mol. of water must be removed from each mol. of chymotrypsinogen at *p*H 2, while at *p*H 3 about 99 mol. of water must be removed to account for the heat changes in the reaction. Since the molecular weight of chymotrypsinogen is 25,000, these dehydrations amount to 5 per cent and 7 per cent respectively. This represents a considerable decrease in hydration since the hydration of proteins in solution seems to be of the order of 20 to 30 per cent. Moreover, certain difficulties in interpreting the effects of *p*H changes and of mineral acid anions lead one to assume that the reversibly denatured chymotrypsinogen formed at *p*H 2 is not identical with that formed at *p*H 3. The cations, for instance, are masked at *p*H 3 by salt bridges with the charged carboxylic groups.[204] But, as in the preceding examples, all these assumptions remain without direct proof; nor must we forget that "no contradiction" does not furnish a sufficient reason for believing that a given hypothesis is true. This is to be born in mind



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especially if several successive assumptions are implied in the same explanation. Also, a "not proved" assumption can never be considered as a valid argument of demonstration. Nevertheless, such tentative interpretations are very useful in showing that the macroscopic modifications observed in reversible denaturation are not due entirely to changes in the protein itself, but are also due to changes in the environment of the protein molecules. Moreover they show that the denaturation processes are not necessarily the same for different proteins or denaturing agents.

*Limiting factors in denaturation reversibility.* As we have seen in the preceding examples, reversal of protein denaturation is in most cases only partial, and this limitation on the reversibility can manifest itself in different ways. Partial reversibility seems often to be due to the existence of two simultaneous competing reactions in denaturation, one of which leads to an equilibrium whereas the other is irreversible. This is shown in a scheme such as: reversibly denatured $\leftrightharpoons$ native $\rightarrow$ irreversibly denatured. In a few cases a more complicated process has been proposed as for instance that the reversible reaction may not be a denaturation reaction. Thus, the hypothetical mechanism of the urea denaturation of diphtheria antitoxin involves two reactions, one leading irreversibly to inactivation of the antibody and the other leading reversibly to an active product. The latter would be more stable in urea than the original antibody and subject to inactivation only by way of slow reconversion to the original antibody.[150]

But, more frequently, the restricted reversibility of denaturation corresponds to successive reactions, the second being irreversible: native $\leftrightharpoons$ reversibly denatured $\rightarrow$ irreversibly denatured. Such a process explains why the loss of activity of trypsin in alkaline solutions is completely reversible for a short time only.[33] Likewise data on the urea denaturation of $\beta$-lactoglobulin are consistent with the view that two stages are involved: the first, which is reversible, is associated with the increase in optical rotation and with the SH group exposure; the second leads to the irreversibly denatured protein insoluble at the isoelectric point.[207] The data seem to conform still better with the diagram $N_s \rightleftarrows N_u \rightleftarrows D \rightarrow D_i$, where $N_s$ and $N_u$ are the stable and unstable forms of the native protein, and $D$ and $D_i$ are the reversible and irreversible states of the denatured protein. The third step is assumed more rapid than the fourth,[225] but, as before, no accurate direct evidence has been given up to the present for any of these transitory molecular states.

Nevertheless, as emphasized by Anson and Mirsky,[23] it is sometimes obscure why it should not be possible, when some reversal occurs, to reverse denaturation completely, at least on repetition of the reversal procedure. With this in mind these authors suggest as a

possible factor causing incomplete reversal, some secondary irreversible change in the solution, which would be a consequence of the denaturation but quite distinct from it. Such a secondary effect, which most frequently seems to be responsible for denaturation irreversibility, is aggregation. It is well established, in the case of chymotrypsinogen for instance, that irreversible denaturation results in an aggregation of the reversibly denatured protein. This is shown by the high concentration dependence of the formation of irreversibly denatured chymotrypsinogen, and is confirmed by ultracentrifugal analysis ($s_{20} = 2{\cdot}2$ for native, $s_{20} = 2{\cdot}15$ for reversibly denatured and $s_{20} = 15{\cdot}7$ for irreversibly denatured chymotrypsinogen). This aggregation depends on the $p$H, temperature, time of heating, protein concentration and electrolyte concentration. Thus irreversible denaturation increases rapidly with $p$H beyond 4, and even at $p$H 3 irreversibly denatured protein is formed when rather dilute solutions are heated between 50 and 60°C for long periods of time. However, at $p$H 3 and 56°C practically no irreversibility is observed if the protein concentration is less than 2 mg per ml., while at $p$H 2 the concentration can be raised to about 4·5 mg per ml. before irreversible denaturation occurs.[204] The part played by aggregation in restricting the reversal of denaturation has been pointed out in processes as different as the salicylate denaturation of haemoglobin,[52] the pressure inactivation of trypsin[187] and the heat denaturation of egg albumin.[79] As we shall see below, such behaviour, showing that very often irreversible denaturation depends on aggregation, may be related to the appearance of the $\beta$-pattern only when the aggregation of denatured molecules can occur; both phenomena are consequences of the same molecular mechanism.

Aggregation is not always connected with the irreversibility of the denaturation, especially if the aggregation is itself completely reversible, as in the case of insulin, where fibril formation suggests the reversible linkage of globular units. Indeed, insulin fibrils may be reverted, by treatment with alkali, into a product similar to native insulin. This reversion is greatly accelerated by increasing the number of available fibril ends, suggesting that disaggregation occurs mainly at these positions. Repulsive forces between similarly charged groups must play a part in the process of fibril dissociation since 1·0 N NaCl inhibits by 90 per cent the reversion.[151] More frequently aggregation is reversible or is at least only partly reversible, as in the cases of chymotrypsinogen[204] and serum albumin;[181] it involves partial reversal of the denaturation. Nevertheless, neither the absence of aggregation nor the complete reversibility of aggregation of the denatured molecules necessarily results in complete reversal of denaturation.



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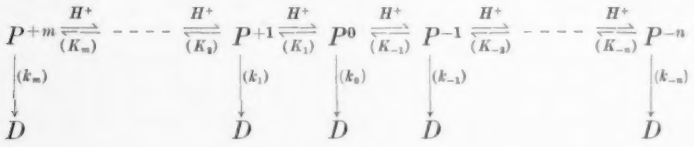
## Intra- and Inter-molecular Mechanisms in Denaturation Processes

We have described and discussed the various effects observed in the reversible and irreversible denaturation of proteins limiting ourselves chiefly to the phenomenological stand-point, with a view to classifying the considerable amount of data sometimes apparently unrelated. The question now arises as to the underlying molecular mechanism, that is to say, the meaning of the denaturation process and of its reversal with respect to the assumed structure of the native molecules. In such a study, the part played by hypothesis is very important and it is necessary to be very cautious in drawing conclusions until we possess exact knowledge about the real molecular structure of proteins in solution. Two types of molecular change are to be considered; intra-molecular changes, that is to say, changes within each protein molecule, and inter-molecular changes, that is to say, changes in the interaction or affinity between different molecules, the latter including the forces between each protein molecule and the solvent molecules as well as those between different protein molecules. These intermolecular changes are direct consequences of the intramolecular modifications. The latter result in denaturation itself, whereas intermolecular changes are usually related to the macroscopic effects of denaturation, such as loss of solubility, and to secondary effects such as aggregation. Moreover, in considering intramolecular modifications, we must discriminate between chemical changes, such as ionization, hydrolysis or the breaking of a bond, and the geometrical or structural changes such as deformation of the polypeptide chains. Both these changes are closely related and are more or less concomitant.

*Chemical aspects of denaturation.* In the earlier studies on denaturation, the molecular change was frequently interpreted chemically. Thus, hydrolysis was postulated to account for heat, acid or alkali denaturation,[6] while esterification accounted for acidic and alcoholic inactivation of insulin.[14] The displacement of the isoelectric point of serum albumin by denaturation seemed to indicate that the protein had undergone a definite chemical change,[24] and likewise the shift of the isoelectric point of wool caused by stretching was assumed to be due to release of the COOH groups.[229] The fact that the addition of sodium caprylate inhibits the urea denaturation of serum albumin had been also considered to prove that caprylate prevents the liberation of active groupings or else combines with certain groups or areas on the protein molecule. It was believed that these same bonds were involved in the initial phases of denaturation.[118], [119] Moreover, only charged molecules can usually be denatured by mild treatments.[81]

The part played by water in denaturation has already been pointed out, in accord with the fact that denaturation is frequently more difficult to effect in the absence than in the presence of water, although the conclusions concerning the changes in hydration caused by denaturation differ according to the various authors, even for the same protein denatured in the same way. For instance, for the heat denaturation of egg albumin, some say that the process is accompanied by an increase in the amount of water associated with each individual protein particle,[25], [26] while others consider that the hydration is less in the heat denatured protein than in the native.[30], [38], [81] This divergence of views is probably due to differences in the $p$H values, although the second point of view is the more generally accepted. Indeed, it is commonly assumed that polar groups, which are free to bind water in native proteins, are coupled in the denatured molecule while non-polar hydrophobic groups are released.[81]

As we have already seen, in the interpretation of the thermodynamics and kinetics of protein denaturation, including the $p$H effects, the part played by hydrolysis or by proton dissociation has been emphasized by several authors. Indeed, the change called denaturation may be only one step in a series of successive processes. In such hypothesis, the general pattern of denaturation may be written:



the values of $m$ and $n$ being, for instance, $m = 2$, $n = 4$ for ricin, $n = 5$ for pepsin, $m = 2$, $n = 1$ for bovine oxyhaemoglobin, $n = 1$ for $\beta$-lactoglobulin and $m = 2$, $n = 4$ for egg albumin.[216] However, it seems that no direct proof has been given of such a hypothesis, and it is difficult to know whether the difference between the activation energy for egg albumin denaturation in neutral media (130 Kcal.) and in acid media (35–37 Kcal.) is due to ionic effects or to the existence of two types of denaturation. The $p$H invariance in the course of the denaturation does not prove that hydrogen ions act only catalytically, since even a one-to-one reaction cannot be detected by $p$H measurements.[215] Moreover, in the case of ricin, if six proton dissociations are considered necessary to fit the data, at least three of these six cannot be identifiable with any titratable groups of the protein. Two are too acid for carboxyl groups while one has a $pK$ with a much more marked temperature dependence that any known prototropic group. It has been proposed that six groups are proton sensitive hydrogen

bonds involved in specific chelations.[189] Nevertheless, it is certain that as the *p*H of a protein solution is changed, ionization or the suppression of ionization of many side-chain acidic and basic groups does occur, such changes certainly affecting the stability of the protein molecule particularly if the dissociation involves certain critical groups. It may be assumed that such a modification is sufficient to bring about considerable lessening of the molecular stability, and to exert a marked effect upon the rate of denaturation.[216] For alkaline denaturation of serum albumin the total number of $OH^-$ ions (whether bound or not) per protein molecule and the percentage of denaturation have been determined at various *p*H values:

| *p*H = | 9·3 | 9·8 | 10 | 10·4 | 10·6 | 11 | 11·4 | 11·6 | 12 | 12·4 |
|---|---|---|---|---|---|---|---|---|---|---|
| $\frac{OH^-}{SA}$ = | 28·8 | 42·7 | 49 | 57·5 | 69·2 | 93·3 | 120 | 148 | 186 | 275 |
| Per cent den. = | 19 | 37 | 42·5 | 54 | 60 | 71·5 | 83 | 89 | 94 | 98·5 |

Thus, 95 per cent denaturation requires 200 $OH^-$, including 150 bound ions, per serum albumin molecule.[250]

Besides the ionization changes, breaking of bonds is frequently considered to be involved in protein denaturation. Moreover, it is sometimes assumed that the process of denaturation is essentially the breaking of bonds, the number broken depending on the conditions.[65] According to different authors, the bonds to be broken are principally salt bridges between the $NH_2$ and COOH groups of the side-chains, and homopolar S-S bonds or hydrogen bonds (for instance 30 hydrogen bonds in the case of egg albumin) between adjacent chains or coils of the same polypeptide chain.[43], [65], [68], [81] Salt bridges have a rather low energy, while the breaking of an SS bond involves 20,000 cal. but only a small entropy change.[169] The hydrogen bond energy is about 6·2 to 7·2 k.cal. for OHO, 6·7 to 8·4 for CHN, 4·1 to 6 for CHO and 6 to 6·8 for NHO.[39], [40] The number of bonds broken in the activation process has been determined for a few proteins: 11 for pepsin, 26 for egg albumin, 15 for haemoglobin, 8 for trypsin. The exact number depends on *p*H; for instance with egg albumin it is 27 at *p*H 7·7 and 19 at *p*H 3·4. In all these processes the contribution of entropy to the free energy of activation is much less than that of enthalpy. Moreover, the number of bonds broken on activation is less than the total number of bonds broken in the complete reaction of denaturation. Thus, in the activation step of trypsin denaturation, 8 bonds are broken whereas 14 are in the total reaction.[169] Generally speaking, there are not enough bonds broken in the activation process to account for the profound physical changes that take place. It is necessary to assume

that the initial step in the denaturation process is the first of a series of chain reactions which lead to the denatured state, the first step involving the simultaneous breaking of bonds and so consuming a large heat of activation. The succeeding reactions are essentially spontaneous, involving the breaking of bonds with small $\Delta H_{\ddagger}^{\ddagger}$ values, while the final reaction produces the precipitable denatured molecule.[149] For the bonds broken first $\Delta H_{\ddagger}^{\ddagger} > T\Delta S_{\ddagger}^{\ddagger}$ while for the last $\Delta H_{\ddagger}^{\ddagger} < T\Delta S_{\ddagger}^{\ddagger}$, whereas if for all the bonds broken $\Delta F_{\ddagger}^{\ddagger} \leqslant 0$, denaturation would be spontaneous and native proteins would be unknown.[65] But, if bonds are broken in the denaturation process, new bonds are probably also built up.[27] Thus, if the heat denaturation of egg albumin obeys 1st order kinetics with respect to protein concentration, the reaction order with respect to time is about 6·0 at 65°C, 4·2 at 70°C and 3·4 at 75°C. Thus the cleavage of hydrogen bonds or salt bridges, which presumably obeys 1st order kinetics, may be followed by the formation of new intramolecular crosslinkages between the polypeptide chains, this second phase of denaturation being of a high order with respect to time.[233] Similarly, in the secondary effects such as aggregation, new bonds are formed between neighbouring molecules by salt bridges, van der Waals forces or hydrogen bonds, and perhaps also in a few circumstances by covalent linkages, chiefly in cases of irreversible aggregation.[166]

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*Structural aspects of denaturation.* As is indicated by all the phenomena described, denaturation results essentially in a structural change in the protein molecules. It has often been considered that denaturation is analogous to the transformation from the crystalline to the amorphous state, although the process of denaturation appears rather to be the modification of a highly specific structure to give rise to a more random arrangement.[19], [40] Such a change in the molecular configuration allows, for instance, the exposure of SH groups, which may be unavailable due to steric hindrance in the native protein.[81] As we have already seen, changes in particle size, caused by dissociation or aggregation, are the most direct manifestation of a structural transformation. Dissociation is quite a frequent phenomenon. For instance, at room temperatures and outside the $p$H range 4–9, the serum albumin molecule splits into smaller particles, the first step (into half molecules) being reversible, while on heating amandin, part of the molecule dissociates into very small particles.[24] Nevertheless, practically nothing is known about the mechanism of such behaviour. As explained above, the mechanism of aggregation has been more fully elucidated, particularly in the case of insulin.[106], [126], [151], [218] Here each molecule undergoes only small structural changes during fibril formation, the globular units being reversibly linked endwise; the heat of dissociation falls with increase of charge. The variation of

viscosity of serum albumin with $p$H in acid media has been interpreted[227] in terms of reversible associations: $A + H^+ \rightleftarrows AH^+$ and $nAH^+ \rightleftarrows (AH^+)n$. More generally, it has been shown that, if denaturation is accompanied by aggregation, the apparent (directly measured) free energy of activation includes a term corresponding to the probability of the particles colliding and aggregating, so that the free energy of activation of the true denaturation may be 3 or 4 times smaller. This is in better agreement with the rather weak perturbation of a protein molecule denatured by mild treatment.[239] But if these denatured molecules are able to aggregate after suitable collisions, this is only because each shows a structure different from that of a native molecule. Thus, it is commonly admitted that native proteins are soluble because most of the side-chains are in protected positions inside of the molecule, whereas the denatured protein coagulates at the isoelectric point because it has a large number of free side chains.[57] The variation of binding ability with denaturation is probably due to the same mechanism. The corresponding change in the configuration of the molecule evidently depends on the folding of the polypeptide chain in the native molecule. Although the existence of polypeptide chains in native protein is still sometimes questioned, the probability of their existence seems high enough to allow us to assume, in the following discussion, that the native protein molecule is made up of one or more polypeptide chains arranged in a specific structure.

Unfortunately, it is not yet possible to decide with certainty the real configuration of the polypeptide chains in the protein molecule. Several more or less complicated models have been proposed from X-ray and infra-red studies, by ASTBURY,[60], [72], [75], [96], [109] PAULING,[199] MIZUSHIMA,[142], [180] ELLIOT,[195], [196], [197] BAMFORD and HANBY,[193], [194] etc. None of these models contradicts the experimental facts, and indeed they fit rather well, as a first approximation, with the observed phenomena. Probably, however, numerous other equally satisfactory models could be conceived, and in the absence of direct proof it seems impossible at the present time to discriminate seriously between the various assumed structures. In all these pictures of the folding of the protein backbone, salt bridges, S-S bonds and hydrogen bonds play an important part in that they stabilize the structure. Nevertheless, facts such as that, of the many proton dissociations demonstrable in ricin, only six need be considered to fit the denaturation data, appear to minimize the importance of salt bridges in determining the structure of the native protein.[189]

Entropy variations during denaturation indicate great disorganization of the molecular structure accompanying the breakdown of bonds other than peptide bonds. All the evidence suggests that denaturation is a series of intramolecular rearrangements leading to

the release of initially coupled side-chains and to the formation of a new and simpler configuration. This denaturation appears as an alteration of the folded native structure corresponding to a more random arrangement. Indeed, the high values of the activation entropy seem to indicate that new degrees of freedom become operative in the activated complex.[50], [57], [65], [69], [101], [189] Nevertheless, according to quantitative immunological techniques, definite structural entities are indicated for denatured protein rather than a disordered state. Thus, although heat denatured egg albumin keeps nothing of the antigenic pattern of native egg albumin, it shows a certain specificity which indicates areas of constant configuration rather than complete structural disorder.[183] From this, as well as from the activation of the peptide linkage, as tested by the biuret reaction, and from the liberation of SH groups, protein denaturation seems to involve an altering or breaking of the secondary peptide linkages.[170] In other words, according to the structural model of PAULING, it seems sound to assume that only secondary and tertiary structures, i.e. intrahelix and interhelix, are involved in the denaturation process without any change in the primary structure of the polypeptide chain.[247] The effect of inhibitors of denaturation would consist in the strengthening of these secondary structures by strong interactions with the stabilizing substances.

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The preceding considerations are valid for globular proteins but not for fibrous proteins like collagen. Indeed, the configuration of collagen chains, which is of very low thermodynamic probability, is maintained only by network forces. On heating in water, the kinetic energy of the chains becomes higher than the cohesion forces, and collagen then loses its fibrous character and undergoes transformation into a globular protein. Such behaviour can be compared with the supercontraction of keratin. Hence the denaturation of fibrous and of globular proteins appear to be opposite phenomena, though both correspond to a large increase in entropy. Consequently, whereas the solubility of globular proteins decreases on denaturation, the solubility of fibrous proteins such as collagen increases.[93]

We have already seen the difficulty of deciding from a phenomenological point of view whether denaturation is an "all-or-nothing" process. The same difficulty arises in considering structural changes. According to the model of ASTBURY, the polypeptide chains, as shown by X-ray patterns, are in the $\alpha$- or the $\beta$-configuration. This is in agreement with the infra-red data, although, as pointed out by LENORMANT, the transformation $\alpha \rightarrow \beta$, as shown by the infra-red spectrum, is hardly ever a total transformation.[243] But this can be explained by considering a mixture of native and of denatured molecules instead of partly denatured molecules. According to the model of

PAULING, the helix of the polypeptide chain may be entirely or only partially unrolled when the weak bonds are broken.[247] Most experimental data seem to indicate, however, that denaturation of a protein molecule occurs in a single jump. This indicates that the folding of the polypeptide chain in the globular molecule is caused by bonds that are mutually dependent[223] so that the loosening of a limited number of these bonds, although not necessarily leading to denaturation, does decrease the amount of energy which must be absorbed to break away from the metastable native configuration. Once past a certain limit, the specific structure of the protein is lost and the polypeptide chain unfolds or rearranges to the denatured state.[189] This explains why denaturation apparently takes place in a single step. From such a standpoint, we see denaturation as a process by which molecules are completely altered and at any given moment there are present only native and completely denatured molecules, there being no gradual change in the molecules throughout the mass of protein.[153] But while this interpretation seems consistent with many observations, it is nevertheless difficult to understand how it can explain that, in a few cases, proteins can show successively reversible and irreversible denaturation, the latter not being due to a secondary intermolecular effect such as aggregation. These difficulties demonstrate that, as long as we have insufficiently precise knowledge of the real internal structure of the protein molecules[237] it is impossible to develop a consistent structural picture of all the different aspects of protein denaturation; only an incomplete glimpse of the phenomenon can be given.

It is commonly assumed, in explaining the increase in viscosity during denaturation as well as the liberation of SH groups and the exposure of peptide linkages to enzymatic proteolysis, that denaturation results in a change from the compact configuration of the native molecule to a more extended and open configuration.[57], [63], [79], [118] Denaturation would consist, therefore, in a more or less complete unfolding of the polypeptide chains.[63], [79], [208], [224], [247] Thus it has been suggested that fully urea-denatured serum albumin consists of two extended polypeptide chains probably held together by the SS groups of cystine residues,[63] although this seems difficult to reconcile with the gelopeptide structure sometimes assumed for egg albumin. As we have seen in discussing the question of particle shape in denatured proteins, an increase in viscosity does not necessarily imply a stretching of the particles. More frequently swelling of the globular remaining particles[240] may be responsible, while the formation of elongated particles by mild denaturation has been shown to be due to the bead-like aggregation of globular molecules which have not undergone any appreciable unfolding of the polypeptide chains.[239] Nevertheless, it seems reasonable to assume that breaking of secondary bonds and the

liberation of initially coupled groups during the course of denaturation are accompanied by a more or less important unfolding of the polypeptide backbone of the molecule. The idea of such partial unfolding agrees with the modifications found in the infra-red spectrum, the appearance of the $\beta$-structure always being due to denaturation.[243] Consequently it seems probable that denaturation results in the $\beta$ transformation (or in any similar transformation) of certain limited parts of the polypeptide chains.

It is important to remark that here, as well as in the following part of this paper, we do not mean by $\alpha$ and $\beta$ forms any precise structures such as $\alpha$-helix of PAULING, the $\beta$-chain of ASTBURY or the $\alpha_{11}$ arrangement of AMBROSE, etc. This reservation is made because we do not know whether these structures, which take no account of the different types of side-chains, really exist in the molecules of globular proteins in solution, nor whether the structure of the molecular backbone is the same in all native proteins. By an $\alpha$ form we imply any structure giving rise to the $6{\cdot}05\mu$ band in the infra-red spectrum, and by a $\beta$ form we mean any structure giving rise to the $6{\cdot}15\mu$ band.

A crucial observation is that the $\beta$ pattern appears only under conditions favouring aggregation, even though precipitation may not occur. The characteristic bands of the $\beta$ form are never observed when aggregation is prevented by a high $p$H value.[243] As it seems difficult to understand how an increase in $p$H can prevent unfolding of the polypeptide chains rather than enhancing it by charge effects, one might think that the $\beta$ structure is stable only after aggregation and even, perhaps, that the $\beta$ structure may be formed only during the course of a concomitant aggregation. Thus, during the denaturation of an isolated protein molecule, the $\beta$ form would be, at the most, a transitory structure between the native $\alpha$ structure and the stable, partially folded form of the denatured single molecule. On this basis, a local $\beta$ structure would correspond to the breaking of specific bonds in the native structure, although no new bonds had yet formed. Depending on the concentration and the $p$H, these new bonds could be formed between parts of the same molecule, when the $\beta$ structure would not be observed, or between different molecules, giving rise to aggregation and thus stabilizing the $\beta$ structure. Association of several molecules can thus occur only when they are momentarily $\beta$ transformed, since the molecular state with a great affinity for similar molecules is a transient state, and we can assume that aggregation is possible only if collisions occur between molecular regions where the chains are in the $\beta$ form. Such a hypothesis may explain the behaviour of protein gels to variations in temperature or $p$H. Indeed, since aggregation proceeds only when collision occurs before the final state of the denatured molecule is attained, the state of the molecules in the aggregates

cannot be stable. If the aggregates are destroyed by heat or by *p*H changes, the alteration of each molecule can be completed up to the final stable state of individual molecules which show no great affinity for other protein molecules.[163]

To interpret completely, from a structural point of view, the behaviour of denatured proteins in solution, it would be necessary to consider more than modifications in the protein molecule itself, and more than changes in the interaction between the solvent and the protein molecules as shown by variations in solubility. One must know about any structural modification (such as freezing) of the solvent itself, as a consequence of the appearance of new groupings on the outside of the protein molecule. Such effects may be very important,[65] although very little information is available on this subject.

According to the preceding rather tentative picture of denaturation, in which not the $\alpha$ form but the $\beta$ form is considered as a metastable state, the reversibility of denaturation may be roughly understood either when aggregation does not occur or when the aggregation can be reversed by heat or *p*H treatment. In the absence of any stabilization by aggregation (or after aggregation reversal), the $\beta$ form, as we have seen, is transformed into a stable denatured form. If, however, the conditions are suitably modified (by cooling, *p*H changes, dialysis, dilution, etc.) before this form is attained, the $\beta$ structure can revert to the $\alpha$ structure, which is very stable in solution, especially if one assumes a model of the type proposed by AMBROSE,[195], [196] or by MIZUSHIMA,[142], [180] which implies a very low entropy. Similarly, one could suggest that, in certain cases, the final denatured molecule is in a state of energy and entropy sufficiently close to that of the initial native molecule to allow reversal to occur with an appreciable probability. Such reversal, either of the unfolding which gives a $\beta$ structure, or of any further chain transformations, implies that there has been sufficient retention of the specific native configuration to assist the return to this state to occur fairly readily in the proper environments.[183], [243], [247] In other words, it implies that the unrolling has not been too drastic, that only limited areas of the native molecules have undergone $\beta$ transformation and subsequent modifications during denaturation. According to this picture of reversible denaturation, the action of agents which reverse denaturation can be compared with the effect of indirect solvents on cellulosic compounds. These solvents introduce themselves between the unfolded segments, and the chains thus liberated can refold into the native form under appropriate conditions.[243]

Such an interpretation also explains the existence of successive or simultaneous reversible and irreversible denaturations; the former generally occurs for only a very short time. Such behaviour can occur

in several cases: when the final denatured form cannot be reversed to a $\beta$ form or to the native form; or if the molecules in the transitory $\beta$ form are assumed to be able to undergo the final transformation by two different routes. One of the latter leads to a reversible form, whereas the other leads to an irreversible denatured state. For instance, this is so if part of the molecules, activated in the $\beta$ form, give rise to reversible aggregation while the other part is transformed directly into irreversibly denatured individual molecules. Or, conversely, it is so if part of the activated $\beta$ molecules undergo irreversible aggregation while the other part is subject to reversible denaturations of the separate molecules. But, as we have emphasized several times, although the preceding hypothesis concerning the intramolecular mechanism of denaturation seems to be quite consistent with all the known experimental data, no direct proof of it has yet been furnished.

## Conclusion

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Without claiming to have exhausted the subject, we have tried, by means of numerous examples, to show the wide diversity of denaturation behaviour and the different aspects of the partial or complete reversibility of the corresponding processes. From this study, it appears that, in spite of a certain similarity between all the observed phenomena, there exist several types of denaturation and reversal depending on the particular protein considered and the particular denaturing agent used. In addition, as a consequence of the extraordinarily great number of properties shown by protein solutions, the extent both of denaturation and of its reversal seems clearly to depend on the criterion chosen to characterize them. Nevertheless, one can safely assume that all these individual and fairly specific effects may be reduced, as a first approximation, to quite a small number of much simpler processes, such as the breaking of hydrogen bonds or salt bridges, the release of reactive groups, proton dissociations, structural changes in the peptide chains, etc. The extent and character of these elementary phenomena depend on the protein species and also on the conditions of denaturation, and it is their simultaneous occurrence which gives rise to what is called protein denaturation.

Moreover, a general qualitative picture of denaturation, whether reversible or not, can be sketched by describing the first steps of this complicated phenomenon in terms of an activation process. According to this hypothesis the activated state of the protein molecule undergoing denaturation can only be stabilized in a metastable state by aggregation: this state is closely related to the $\beta$ form. In the absence of aggregation and depending on the conditions of temperature, $p$H and chemical environment, this transitory molecular state can either

proceed reversibly back into the initial native state or may be transformed into a stable denatured state. The different kinds of denaturation reversal can be interpreted successfully on this basis. In the absence of more accurate and detailed knowledge of protein structure, it is still impossible to give a quantitative structural description of the denaturation process for any given protein under given conditions. We must, therefore, restrict ourselves to a tentative and qualitative interpretation of the experimental data.

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